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**Evaluation of pre- and post-vaccination Influenza A Virus antibody responses in  
breeding age replacement gilts from twelve production systems in the United States**

by

**James Scott Carlson**

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Microbiology

Program of Study Committee:  
Phillip Gauger, Major Professor  
Jianqiang Zhang  
Amy Vincent

The student author and the program of study committee are solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2017

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## DEDICATION

To my grandmother who helped support, house, and motivate me during my graduate career. I would not be the man I am today without your guidance. Thank you.

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## ABSTRACT

Influenza A virus (IAV) is an important respiratory pathogen of swine in the United States. Whole virus, inactivated vaccines are a common method used to control infection and clinical disease. However, homologous IAV vaccine antibody responses are rarely evaluated in the field and many influenza serological evaluations are lacking. New replacement gilts are an unstudied population in breeding farms and have been shown to affect viral ecology through introducing new IAV or lacking protection against resident viruses. Serological evaluations are uncommon in the current literature and could prove important in evaluating vaccine responses. This study sought to address all of these points by conducting a one-year longitudinal serological evaluation of replacement gilts on twelve different United States farms.

The study was performed by acquiring serum samples from each farm at four time points over the course of a year. The serum samples were evaluated using nucleoprotein enzyme-linked immunosorbent assay and hemagglutination inhibition assay. The hemagglutination inhibition assay was performed using homologous vaccine antigens based on each farm-specific vaccination protocol and six representative viruses, which were selected due to their predominance in the US swine population and representing the major antigenic clades and genetic clusters.

The study found that a large number of gilts were IAV antibody negative when delivered to the breeding farm or at the age when gilts are breed for production. This suggests new replacement gilts might be susceptible to endemic IAV and may help maintain endemic infections on a farm. It was also found that the levels of vaccine-induced antibody

were highly variable between farms and overtime on the same farm. This variability in the number of gilts demonstrating homologous vaccine antibody responses suggests gaps in protection may occur which allows for either the maintenance of endemic viruses within a herd or creates a risk of lateral infection with new or emerging IAV from outside sources. This study supports the idea of increased serological surveillance to evaluate vaccine antibody responses that may correlate with efficacy and help determine the magnitude of protection on a farm or reasons why vaccines appear to fail if antibody responses are lacking.

## **CHAPTER 1. INTRODUCTION**

### **Statement of the Problem**

Replacement gilts are an important subpopulation of swine in breed-to-wean production systems replacing approximately 50% of the breeding herd each year in the US. Therefore, new gilts entering the breeding herd may negatively influence disease dynamics in a production system including IAV. Therefore, it is important to understand the IAV status of new replacement gilts, including the presence of IAV antibody, prior to entry into the breeding herd. In addition, protecting new gilts against endemic IAV through vaccination is an important biosecurity method incorporated into isolation and acclimation protocols. Evaluating the IAV vaccine antibody response in new gilts may help identify potential reasons for perceived vaccine failures and suggest areas that may help improve our methods to control IAV in breed-to-wean production systems.

### **Objectives**

The first objective of this field study was to evaluate the presence of influenza A virus (IAV) antibody in a cohort new replacement gilts selected from twelve different swine farrow-to-wean production systems in the United States (US) coinciding with isolation/acclimation and prior to IAV vaccination. The second objective was to conduct a longitudinal study to evaluate the IAV vaccine antibody response for approximately one year that included the post-vaccination and post-integration time points using a subset of the same replacement gilts. Replacement gilts were the target subpopulation due to their potential influence on the IAV dynamics in breeding

herds that may include introducing new IAV, supporting endemic infections, or representing an IAV immunologically naïve subpopulation within the production system.

## **Thesis Organization**

This thesis is organized into five chapters. The first chapter is a general introduction to the thesis. The second chapter consists of a literature review summarizing IAV with an overview of serology data available for swine in North America. The third chapter evaluates the IAV antibody status of non-vaccinated, new replacement gilts of breeding age. The fourth chapter evaluates the presence and magnitude of the IAV antibody in a subset of the same gilts for approximately one year after IAV vaccination and integrating into the main breeding herd. The fifth chapter is a summary of the IAV antibody data that was observed during the study.

## CHAPTER 2. LITERATURE REVIEW

### Introduction

Influenza A virus (IAV) is a primary cause of respiratory disease in swine worldwide and can be a source of zoonotic infection in humans (Vincent et al., 2008). Currently, three major subtypes of IAV, H1N1, H1N2, and H3N2, co-circulate in North American swine (Vincent et al., 2008). The predominant surface proteins, hemagglutinin (HA) and neuraminidase (NA), are responsible for viral attachment and release in permissive cells, respectively, and provide partial restriction of their host range. The HA protein represents considerable genetic and antigenic diversity complicating the ability to prevent infection and clinical disease using traditional biosecurity practices and commercial or farm-specific inactivated vaccines (Vincent et al., 2008). Currently the HA of the H1 subtype consists of eight phylogenetic clades that includes:  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\gamma$ 2,  $\delta$ -1a,  $\delta$ -1b,  $\delta$ -2, and pandemic (Vincent et al., 2014). There are eight H3 phylogenetic clusters as follows: cluster IV, IV-A, IV-B, IV-C, IV-D, IV-E, IV-F, and human-like (Kitikoon et al., 2013). The most prominent clades in North America are the H1  $\gamma$ , H1  $\delta$ -2, and H3 cluster IV-A (Vincent et al., 2017). Influenza vaccines in swine have become more common, particularly in breeding swine, to prevent clinical disease but do not prevent infection (Corzo et al., 2012). A complication of increased vaccine use is the difficulty interpreting IAV serology outcomes. Serology tests are unable to differentiate between antibody induced by natural infection, IAV vaccines, and maternal antibody that can be passively transferred from naturally infected or vaccinated dams (Loeffen et al., 2003). Historically, the hemagglutination inhibition (HI) assay was the gold standard for detecting IAV antibody and determination of the HA subtype (Yoon et al., 2004). However, increasing IAV genetic and antigenic diversity has complicated our ability

to effectively use HI assays to evaluate IAV antibody responses in swine. Recently, the use of enzyme-linked immunosorbent assays (ELISA) based on the conserved nucleoprotein (NP) antigen has become more common for detecting IAV immune responses although this assay also does not distinguish between natural infection and vaccine antibody (Ciacci-Zanella et al., 2010; Goodell et al., 2016). Additionally, some new vaccine platforms, like replicon particle vaccines, do not induce an NP antibody response and thus cannot be evaluated using the NP ELISA (Vander Veen et al., 2012). Collectively, the diverse IAV ecology circulating in swine has altered how veterinarians and animal health officials use serology tests to answer specific diagnostic questions and interpret the results.

Replacement gilts have become an important subpopulation in breed-to-wean farms that may influence the disease dynamics in a production system. Disease monitoring is often minimal in new replacement gilts and limited to detection of porcine reproductive and respiratory syndrome virus (PRRSV). New gilts may impact the epidemiology and ecology of IAV in breeding herds based on their status upon entry to the farm. Monitoring the presence and magnitude of IAV antibody as well as the virus may be an important biosecurity method in the overall control of influenza in breed-to-wean production systems.

### **Influenza A Virus Classification And Genome Organization**

Influenza A Virus, a member of the *Orthomyxoviridae* family, is a negative sense, single stranded, RNA virus (Zell et al., 2013). The virus contains an envelope and eight genome segments that code for 11-12 proteins (Sandbulte et al., 2015; Zell et al., 2013). The HA and NA proteins are encoded on Segment 4 and 6, respectively, of the viral genome (Lorusso et al., 2011). Influenza A viruses are subtyped based on classification of their HA and NA genes.

Currently, 18 HA subtypes and 11 NA subtypes have been detected circulating in nature, but only a small subset are endemic to swine (Sandbulte et al., 2015; Urbaniak and Markowska-Daniel, 2014). The additional genetic segments comprising the internal structural and non-structural proteins are as follows: Segment 1 = Polymerase Basic 2 (PB2), Segment 2 = Polymerase Basic 1 (PB1), Segment 3 = Polymerase Acidic (PA), Segment 5 = Nucleoprotein (NP), Segment 7 = Matrix 1 (M1) and Matrix 2 (M2), Segment 8 = Non-structural 1 (NS1) and Non-structural 2/Nuclear Export Protein (NS2/NEP) (Lorusso et al., 2011).

The HA protein is primarily responsible for binding to host cells, internalization of the virus, and endosomal membrane fusion once inside the permissive host cell (Steinhauer, 1999). Internalization and membrane fusion are accomplished by cleavage of the HA protein which requires host proteases. The availability of proteases to cleave the HA protein determines host and tissue specificity for IAV (Matrosovich et al., 2009). For example, IAV is able to target the gastrointestinal tract in avian species due to the presence of proteases in enterocytes with the ability to cleave the HA protein (Thacker and Janke, 2008). In contrast, mammals contain proteases capable of cleaving the HA protein that are exclusive to the respiratory tract (Janke, 2014; Lipatov et al., 2008). The HA protein targets specific receptors by binding to sialic acid moieties located on the surface of host cells. Mammalian influenza viruses target  $\alpha$ 2-6 sialic acid moieties and avian influenza viruses target  $\alpha$ 2-3 sialic acid moieties (Matrosovich et al., 2009; Paulson, 1985; Sandbulte et al., 2015). The HA protein is also the target of neutralizing antibodies that can prevent attachment to the host cell receptor or inhibit cleavage of the HA protein once inside the endosome, thus preventing membrane fusion, viral entry, and subsequent replication (Van Reeth and Ma, 2013; Wiley et al., 1981).



The NA protein is responsible for releasing nascent virus from the host cell by cleaving the attachment between sialic acid residues and HA protein using an intrinsic sialidase (Reperant et al., 2012; Urbaniak and Markowska-Daniel, 2014). Mutations in the NA gene can alter viral virulence by affecting transmission through the ability of IAV to exit host cells (Janke, 2014; Reperant et al., 2012). The inability to successfully exit an infected cell causes the virus to become vulnerable to other innate immune responses, such as apoptosis and phagocytosis, as the virus becomes trapped within the dying cell instead of escaping to find a new host cell (Reperant et al., 2012).

The polymerase genes, PB1, PB2, and PA, are responsible for replication of the viral genome and hijacking the host cellular machinery. All three proteins work together to form the viral RNA dependent RNA polymerase (Basler and Aguilar, 2008; Janke, 2014). PB1 is the catalyst for polymerization whereas PB2 and PA are responsible for stealing the 5' cap of host mRNA (Janke, 2014; Urbaniak and Markowska-Daniel, 2014). All three polymerase subunits are packaged with the viral nucleoprotein as a complex and become activated once the virus has entered a new host cell (Basler and Aguilar, 2008; Janke, 2014).

The non-structural protein I (NS I) interferes with host cell signaling that inhibits the production of antiviral cytokines including Interferon  $\alpha$  and  $\beta$ , Tumor Necrosis Factor  $\alpha$ , and Interleukin 6 (Basler and Aguilar, 2008; Janke, 2014).

The M2, or Matrix 2, is an ion channel protein incorporated into the viral envelop. After the virus is endocytosed by the host cell, the virus is placed in an endosome which is acidified by host ion pumps. The M2 protein then pumps these ions into the viral capsid to lower the pH (Samji, 2009). Once the pH is lowered beyond a threshold, it triggers the release of the viral

genome from the viral M1 proteins and the viral RNPs are released into the cytoplasm. Once in the cytoplasm, the viral genome begins replication (Sandbulte et al., 2015).

All IAV genes have been shown to influence virulence, either individually or in combination with one another (Janke, 2014). A functional virus contains eight ribonucleoprotein (RNP) complexes, which is a composite of the viral RNA and NP. The eight RNPs are encapsulated together with the M1 protein in a viral capsid (Samji, 2009). The capsid is subsequently covered by the viral envelope, which is derived from the host cell membrane and includes the HA, NA, and M2 proteins (Lee and Saif, 2009; Samji, 2009). A single RNP complex consists of an individual RNA genome segment that encodes one or more viral proteins, the viral NP, which binds to and protects the viral RNA, and the viral polymerase complex consisting of the PA, PB1, and PB2 proteins (Lee and Saif, 2009). Upon entry, the RNP complexes are released into the host cell when the host and viral membrane fusion occurs. The RNP complexes contain nuclear localization signals and begin viral genome replication upon reaching the nucleus (Reperant et al., 2012).

Influenza viruses change and evolve over time due to two main mechanisms: genetic drift and genetic shift (Zell et al., 2013). Genetic drift results from the accumulation of small point mutations in key regions of the HA gene can alter the antigenic properties of the virus (Zell et al., 2013). Genetic shift is the result of a process known as reassortment. Reassortment can occasionally occur when two or more strains of IAV co-infect a single host cell and then exchange one or more genome segments (Urbaniak and Markowska-Daniel, 2014). Reassortment between any of the viral genes can lead to the development of novel strains with altered host specificity and virulence characteristics (Sandbulte et al., 2015; Zell et al., 2013). Many swine viruses are the result of previous reassortment between human or avian viruses with endemic

swine viruses which may produce a novel and virulent virus that is easily transmitted in a swine population if cross-protective immunity is partial or absent (Brown et al., 1998; Diaz et al., 2017; Vincent et al., 2009; Winkler and Cheville, 1986). Since each of the eight viral gene segments act independently, there are 256 possible different genetic combinations that could possibly result from two different strains of IAV reassorting. Fortunately, many reassortant viruses fail to establish endemic infections within a swine population (Urbaniak and Markowska-Daniel, 2014).

## **Epidemiology**

### *North American IAV Genetic Lineages*

Surveillance of swine herds is performed through a passive process by the United States Department of Agriculture using porcine submissions to the National Animal Health Laboratory Network (NAHLN) member diagnostic laboratories (Sandbulte et al., 2015). Veterinarians voluntarily submit diagnostic samples from swine with clinical respiratory disease to diagnose a cause. If IAV is detected, subtyping and virus isolation are performed with sequencing of successful virus isolates. The viral isolate and data are then submitted to the National Veterinary Services Laboratory (NVSL) repository that is maintained for public use. The population dynamics of IAV-S over the last several years have been monitored using the data from the USDA surveillance program (USDA, 2017).

Reports based on the USDA data have determined that there are 16 distinct H1 and H3 genetic clades of IAV in US swine (Anderson et al., 2015; Kitikoon et al., 2013; Vincent et al., 2014; Vincent et al., 2017). The classical H1N1 virus is thought to have originated from the 1918 Spanish Flu human pandemic, which spilled-over into North American swine. The virus remained antigenically stable in swine for 80 years, from 1918 to 1998, when the H3 subtype

became established in swine (Sandbulte et al., 2015; Vincent et al., 2008). Classical H1N1 was first diagnosed in 1919 and later isolated in the 1930s (Shope, 1931). Currently, the H1 subtype has emerged into multiple phylogenetic clusters in North America designated as  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\gamma$ -2,  $\delta$ -1a,  $\delta$ -1b,  $\delta$ -2 and the H1N1 pandemic (H1pdm09) (Lorusso et al., 2013; Vincent et al., 2009). The  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\gamma$ -2 clades are descendants from the classical H1N1 and typically have swine origin HA and NA genes (Lorusso et al., 2011; Vincent et al., 2014). The  $\delta$ -1a,  $\delta$ -1b and  $\delta$ -2 H1 viruses descended from human-origin viruses (Anderson et al., 2013; Sandbulte et al., 2015). The  $\gamma$  and  $\delta$  H1 clades have virtually replaced the  $\alpha$  and  $\beta$  H1 clades in North American swine (Chen et al., 2012).

In the 1990s, a new virus of the H3 subtype emerged in US swine. The H3 became established in swine from a reverse zoonotic event where a human seasonal H3 crossed the species barrier and infected swine (Zhou et al., 1999). These reverse zoonotic events may occur when humans and swine are in close proximity, such as employees at swine farms or exhibition shows and fairs (Feng et al., 2013). The 1998 H3N2 virus contained the triple reassortant internal gene (TRIG) cassette and has since produced many reassortant progeny IAV (Lorusso et al., 2011; Vincent et al., 2014). The TRIG cassette was the result of a reassortment event between human, avian, and swine lineage viruses that resulted in the following: human origin PB1 gene; avian origin PA and PB2 genes; swine origin NS, NP, and M genes (Vincent et al., 2008; Webby et al., 2000). The TRIG cassette has the ability to acquire a variety of HA and NA genes in swine IAV. Currently, the TRIG is detected in all contemporary strains of both H1 and H3 subtypes (Lorusso et al., 2013; Vincent et al., 2008).

Spillover events from human viruses have repeatedly influenced the genetic diversity of influenza A viruses in swine (IAV-S). In 2005, a reverse zoonosis event occurred involving the

H1 subtype, which lead to the development of the  $\delta$ -cluster IAV (Lorusso et al., 2013). The  $\delta$  cluster has become highly prevalent in North America and can be found in US and Canadian swineherds (Choi et al., 2002b; Karasin et al., 2006). The  $\delta$ -cluster can be subdivided into the  $\delta$ -1 and  $\delta$ -2 clusters with putative  $\delta$ -1a and  $\delta$ -1b clades that have become established (Anderson et al., 2015; Choi et al., 2002a). The  $\delta$ -1 and  $\delta$ -2 clusters are the result of a different reverse zoonotic event and each prefers a different NA subtype;  $\delta$ 1 prefers N2 subtype and  $\delta$ 2 prefers N1 subtype (Lorusso et al., 2013; Vincent et al., 2009).

A more recent phylogenetic cluster to emerge is the H1pdm09. The H1pdm09 is a swine-lineage reassortant IAV that was first identified in humans in Mexico in 2009 (Mena et al., 2016; York and Donis, 2013). The virus quickly spread worldwide. The H1pdm09 has been detected in most major swine populations, such as the US, China, Argentina, and Germany, and is worldwide (Dibárbora et al., 2013; Liu et al., 2011; Nelson et al., 2015a; Nelson et al., 2015b). It is still unclear where the virus originally evolved. Antigenic and genetic characterizations have failed to pinpoint the origins of the virus due to gaps in the global surveillance data (Vincent et al., 2014; York and Donis, 2013). It has been theorized that the IAV developed from precursor viruses in Mexico with subsequent reassortment with IAV from Asia that were present in imported swine (Mena et al., 2016). The pdm09 virus contained six gene segments from a North American triple reassortant swine-lineage (PB2, PB1, PA, HA, NP, and NS) and two gene segments from a European swine-lineage (NA and MP) (York and Donis, 2013). The H1pdm09 virus has reassorted with many endemic strains in swine to generate novel reassortant viruses (Kong et al., 2015). The H1pdm09 strain matrix (M) gene is currently detected in all US strains of IAV-S regardless of subtype or cluster (Kong et al., 2015; Nelson et al., 2015a; Sandbulte et al., 2015).

One of the most recent human to swine spillover events was the emergence of a novel human-like H3 (H3 hu-like) in swine from the 2012 season (Rajão et al., 2015). Veterinary diagnostic laboratories began detecting the H3 hu-like during the 2012 season and have consistently recovered a small number each of the following years (USDA, 2017). In 2015, a study was performed to analyze the phylogenetic relationships of two isolates of the novel H3 hu-like (Rajão et al., 2015). It was determined that the HA gene was related to the 2010 human seasonal IAV, the NA was also derived from the 2002 human seasonal IAV, and the internal genes were a mix of the TRIG constellation and H1pdm09 internal genes (Rajão et al., 2015). Interspecies transmission events continue to occur and lead to greater genetic diversity in IAV-S (Nelson et al., 2012).

### **Clinical Features**

Traditionally, IAV-S is a seasonal disease that peaks during the cold winter months (Liu et al., 2016). Currently, IAV-S infections can be detected throughout the year and in all age groups of swine due to the increased genetic diversity of different strains of IAV (Vincent et al., 2014). Influenza has high morbidity, upwards of one hundred percent, and low mortality, often less than one percent. These factors can vary between strains and within populations (Vincent et al., 2014). The virus is primarily spread through pig-to-pig contact via the nasopharyngeal route. The virus is shed from the nose or mucus and spreads via aerosols and droplets (Brown, 2000).

Typical clinical signs include coughing, sneezing, loss of appetite, nasal congestion, nasal discharge, and lethargy. Symptoms typically begin 1-2 days post infection and continue for 7-10 days, with viral clearance within 2 weeks or less with some variation (Brown, 2000; Liu et al., 2016). High fevers are common during the second and third days post infection, but often abate

as more typical clinical signs develop around the fourth or fifth day post infection (Janke, 2013). The disease has a short incubation time of 2-3 days and as a result, whole herds will often contract the disease within a span of a few days depending on population immunity (Janke, 2013). Infected swine do not always demonstrate clinical signs suggestive of influenza. Many pigs can have a sub-clinical infection where they shed virus but do not have signs of distress or disease (Corzo et al., 2013). As a result, clinical observations are not always a reliable method to diagnose influenza in a swine population (Vincent et al., 2014). Virus shedding typically begins 1-2 days post infection and can continue for up to one week, which also contributes to the rapid spread of the virus throughout a herd. One study, performed in Spain during 2008/09, reported over 90% of farms were positive for IAV-S via serological tests but only about 9% of those farms reported observing clinical signs of disease in their swine (Simon-Grifé et al., 2011).

IAV primarily targets the respiratory tract in swine and viral replication and tissue damage occur in the epithelium of the respiratory tract (Janke, 2014). The virus infects epithelial cells from the nasal mucosa to the alveoli of the lungs. In the lungs, it causes mild to severe microscopic lesions that result in necrotizing bronchitis and bronchiolitis (Janke, 2014; Winkler and Cheville, 1986). Within the first 48 hours post infection, the epithelium begins to slough off as cells die due to viral replication and immune-mediated damage (Janke, 2014). Viral replication directly causes cell death by suppressing host cellular functions and inducing cell lysis. Cell death induces an inflammatory response due to cytokine mobilization, which causes further damage to the tissues in the lungs (Oldstone et al., 2013).

The immune response to influenza infection can be divided into two steps: the innate and the adaptive responses. The innate response starts when immune cells recognize broad features that are common to viral pathogens, such as double strand RNA replication intermediates

(Yoneyama et al., 2004). During the first 24 hours post infection, neutrophils migrate to the site of infection and begin secreting pro-inflammatory cytokines and chemokines. After 24 hours post infection, macrophages begin to replace neutrophils as the primary immune cell (Janke, 2014). Macrophages and dendritic cells engulf viruses, or virus infected cells, and then present the antigens to adaptive immune cells in the lymph nodes (Braciale et al., 2012). During a cytokine storm, which is an over production of cytokines, vascular leakage and cell death may occur, which causes tissue damage (Janke, 2014). Cytokines and inflammation are necessary for viral clearance but the immune system must maintain a careful balance to prevent tissue damage that could impair lung function (Oldstone et al., 2013). If too much tissue damage occurs in the lung, it may lead to respiratory failure (Braciale et al., 2012). As a result, many immune cells also secrete anti-inflammatory compounds to modulate the host immune response.

The adaptive immune response has two important components: humoral and cell-mediated immunity. The humoral component involves induction of antibodies against specific proteins of the virus to prevent infection and transmission, also known as neutralizing antibodies (Waffarn and Baumgarth, 2011). Antibodies are produced by B-cells and target specific regions of viral proteins called epitopes (Sandbulte et al., 2015). The HA protein of IAV is the target for neutralizing antibodies (Sandbulte et al., 2015). After clearance of the virus, some B-cells mature into memory cells which can be rapidly induced during subsequent infections (Van Reeth and Ma, 2013). Antibody peaks at two to three weeks post infection and then slowly wanes over time (Larsen et al., 2000). The cell-mediated component involves mobilizing T-cells. Cytotoxic T-cells are able to recognize and kill infected host cells that display viral proteins on their surface, which helps clear the virus from the body (McMichael et al., 1983; Sandbulte et al., 2015; Van Reeth and Ma, 2013).



Macroscopic lesions develop in the lung due to viral replication and immune responses resulting in tissue damage. These lesions are usually most prominent in the cranioventral portions of the lung and can range from 10% to upwards of 40% of the pulmonary parenchyma depending on the severity of the infection (Janke, 2013). The tissue damage is often multifocal, meaning areas of lung damage can be adjacent to unaffected tissue, and are scattered throughout the cranioventral regions of the lung often in a lobular pattern. This is true at both the macroscopic and microscopic levels (Janke, 2013). By two weeks post infection, the virus is cleared and lung tissue has recovered from infection (Janke, 2013). Infection rarely causes permanent lung damage and recovery is often complete. However, severe infections complicated by secondary bacteria may cause permanent lung damage leading to decreased respiratory efficiency (Janke, 2014).

### **Detection and Diagnosis**

There are multiple methods available to demonstrate IAV infection in swine. One method is to directly isolate the virus from samples collected from animals, such as nasal swabs (Goodell et al., 2013). Another method is to test samples by PCR for the presence of viral genetic material, such as tissue samples, nasal swabs, and oral fluids (Zhang and Harmon, 2014). The caveat for both of these tests is that they require an active, acute infection in order to detect the virus. This is problematic since the window for influenza infection is short, about 7-10 days, and the window for viral shedding is even shorter, often only 3-5 days (De Vleeschauwer et al., 2009; Janke, 2013; Sandbulte et al., 2015; Vincent et al., 2006). Serological or antibody based assays are not as limited by the duration of infection. Once an animal has developed an immune response to a pathogen, it often lasts several weeks (Larsen et al., 2000; Markowska-Daniel et

al., 2011; Sreta et al., 2013). This allows for serological testing and evaluation long after an infection has occurred. As a result, serological assays are highly useful for detecting previous exposure on the individual level and the prevalence of exposure within a population, although it should be noted that vaccination can alter the antibody profile in vaccinated animals and thus complicate the interpretation of serological assays.

#### *Nucleoprotein Enzyme-linked Immunosorbent Assay*

The Nucleoprotein Enzyme-linked Immunosorbent Assay (NP ELISA) is a diagnostic test used to detect antibodies against the nucleoprotein of IAV. The NP is a highly conserved protein across IAV strains thus the NP ELISA is a broad test used to detect influenza exposure in multiple species (Goodell et al., 2016). Anti-NP antibodies are not a measure of neutralizing ability, thus they do not correlate with protection. Natural infection and whole virus vaccination typically induce the development of anti-NP antibodies in swine (Ciacci-Zanella et al., 2010). The NP ELISA is able to detect anti-NP antibodies from natural infection, whole virus vaccination, and maternally derived antibodies that have been passively transferred to offspring from naturally infected or vaccinated dams (Ciacci-Zanella et al., 2010). This assay cannot provide information on subtype of the virus, nor can it differentiate between natural exposure, passive immunity, or vaccine antibody (Goodell et al., 2016).

The Multi-Screen NP ELISA (IDEXX, Westbrook, ME) is a blocking ELISA and was used by Goodell et al., 2016, to evaluate antibody presence in swine serum (Goodell et al., 2016). The ELISA kit comes with a 96-well plate coated with IAV NP antigen based on a specific, conserved epitope in the protein. Serum is added to the plate and allowed to incubate, followed by a control antibody with a reactive tag. Post-incubation, a washing step is performed to remove

unbound antibody. Next, a chemical solution is added that reacts with the tag if present, and finally the plate is analyzed by a plate reader to determine the optical density of the color change. The values for each sample are compared to a negative and positive control and a Sample/Negative ratio is created. A 0.6 cut-off value is suggested by the manufacturer to determine positive and negative results. Values  $>0.6$  are considered negative and results  $<0.6$  are considered positive. Previous work has shown that the NP ELISA is very sensitive and specific (Goodell et al., 2016).

### *Hemagglutination Inhibition Assay*

The hemagglutination inhibition (HI) assay is the preferred method for detecting and quantifying a protective antibody response to a specific strain of IAV (Dibárbora et al., 2013). The assay is relatively inexpensive and easy to perform as it only requires red blood cells (RBC), a reference virus, and the serum sample to be tested (Detmer et al., 2013). However, serum treatment is mildly time-consuming and necessary to remove non-specific serum agglutinins and inhibitors of agglutination (Kitikoon et al., 2014). The assay is performed by adding serum, treated and diluted 1:10, in a series of two fold dilutions in a 96-well plate, to which the virus antigen is added to each well, except the control well. The virus is diluted to a working concentration of 8 HA units (Detmer et al., 2013). The serum and virus are allowed to incubate for at least 30 minutes followed by the addition of 0.5% solution of RBCs to each well. Antibodies present in the serum will prevent RBC agglutination (Kitikoon et al., 2014). This results in a clear well with a compact “button” of RBCs at the bottom of the well. If no HI antibodies are present, the virus will bind to the sialic acid receptors on the surface of the RBCs and form a crosslinking lattice within the well. This lattice will produce a cloudy or hazy

appearance in the well known as agglutination (Kitikoon et al., 2014). The HI titer is the inverse of the highest dilution that displays inhibition. Titers greater than or equal to 40 are known to be protective and correspond to a 50% reduction in infection for an individual (Coudeville et al., 2010; Hancock et al., 2009; Hobson et al., 1972).

### *Serum Virus Neutralization Assay*

The serum virus neutralization assay (SVN) is another less common serological method of evaluating the functional antibody titer in a serum sample (Truelove et al., 2016). Serum is serially two-fold diluted in a 96 well plate and mixed with a known concentration of virus which is allowed to incubate for at least one hour before adding to a monolayer of MDCK cells and allowed to incubate for up to 48 hours (Gauger and Vincent, 2014). The cell culture plate is then evaluated for cytopathic effect (CPE) and wells without CPE are considered positive for neutralizing antibodies. Relative antibody titer is the reciprocal of the highest dilution without CPE (Detmer et al., 2013). SVN assays are useful because they demonstrate neutralizing antibodies to a variety of epitopes that can prevent viral infection of permissive cells (Gauger and Vincent, 2014). The SVN assay also requires more time, up to 48 hours compared to the HI assay, and reagents to perform the test, such as Madin-Darby Canine Kidney (MDCK) cells. The HI and the SVN titers are correlated in most cases for IAV (Truelove et al., 2016).

### **Vaccination and Prevention**

The goal of a vaccine is to induce a protective immune response (Vincent et al., 2016). The protective immune response helps reduce viral shedding, severity of lung lesions, and influenza-like illness. Ideally, the immune response becomes a memory response that will be

protective against future infections if the virus is antigenically similar to the vaccine antigen (Chen et al., 2012). An ideal vaccine would provide complete protection and prevent shedding, lung lesions, and clinical signs of infection upon exposure to a homologous virus (Loving et al., 2013). The challenge is to create a vaccine that extends that protection to heterologous IAV (Chen et al., 2012). There are several types of IAV vaccines including inactivated virus (commercial and autogenous), modified live virus / live attenuated virus, DNA based vaccines, subunit, and vectored (Chen et al., 2012).

Inactivated whole virus and autogenous vaccines are killed versions of a virus administered to stimulate the immune system. The virus is rendered non-infectious by either chemical, thermal, or radiation based inactivation (Chen et al., 2012; Platt et al., 2011). This type of vaccine is often administered intramuscularly and induces a robust humoral response but has demonstrated limited cross protection against heterologous strains (Chen et al., 2012; Van Reeth and Ma, 2013; Vincent et al., 2016). The lack of cross protection may be due to limited mucosal immunity that is weakly primed with a vaccine administered only through the intramuscular route (Heinen et al., 2001; Van Reeth and Ma, 2013). Inactivated whole virus vaccines are often multivalent to induce broader protection but each additional virus added increases production costs (Van Reeth and Ma, 2013). Multivalent vaccines are necessary due to the increased viral diversity and poor cross-reactivity observed with inactivated vaccines (Lee et al., 2007).

Autogenous vaccines are developed from an IAV isolated from the farm where the virus is currently circulating (Sandbulte et al., 2015). Theoretically, a vaccine developed from a farm-specific viral isolate will provide superior protection compared to a commercial product that may not antigenically match the endemic strains on a farm. Autogenous vaccines have less regulatory

hurdles but restricted to use on the farm where the vaccine virus was isolated (Ma and Richt, 2010).

Live attenuated influenza virus (LAIV) vaccines or modified live virus (MLV) vaccines use an attenuated, or replication deficient, virus to prime the host immune response. A common method of attenuation is to mutate one of the viral genes, such as NS1 or one of the polymerase genes, which are responsible for altering host cytokine response or viral replication, respectively (Richt et al., 2006; Van Reeth and Ma, 2013). Without fully functional genes, the virus is unable to sustain an infection in a healthy host and only replicates a few times before the host immune system is able to clear the infection well before clinical disease would develop. Live attenuated vaccines are able to induce more cross-reactive immune responses compared to inactivated vaccines due to induction of both humoral and cell-mediated immune responses (Vincent et al., 2007). One concern with live attenuated vaccines is the potential for reassortment with wild type viruses. Another concern is the potential for a live attenuated vaccine to revert back to a virulent virus (Chen et al., 2012). While both concerns are unlikely to occur, live attenuated vaccines have not been approved for use in swine until 2017 with the launch of the first LAIV vaccine in swine known as Provenza produced by Boehringer Ingelheim Vetmedica (Loving et al., 2013).

DNA Vaccines are plasmids, which encode viral proteins that are injected into hosts to induce an immune response. Upon entering a host cell, the vaccine plasmid expresses the viral proteins encoded on the plasmid, thus priming the host immune system to those proteins (Chen et al., 2012; Kim and Jacob, 2009). DNA vaccines are non-infectious, can be made multivalent by adding additional viral genes, and induce both cellular and humoral immune responses (Dhama et al., 2008). The complex and laborious process of integrating a plasmid into a host cell is the

main drawback of DNA vaccines (Olsen, 2000). DNA vaccines also have short-lived immune responses and thus require periodic boosts to maintain protective levels.

Subunit vaccines include the protein components of the virus which are administered with an adjuvant to induce an immune response, primarily humoral (Chen et al., 2012). The advantages of subunit vaccines are the ability to target the immune system against specific neutralizing antigens (HA protein), the ease of including multiple antigenic variants in one vaccine, and the proteins are non-infectious (Chen et al., 2012). The difficulty with this vaccine platform is producing the viral proteins, which often requires the use of a transgenic vector organism (D'Aoust et al., 2008). In addition, pure proteins are not immunogenic and an adjuvant is necessary to induce an adequate immune response (Chen et al., 2012). A key consideration is picking the correct antigenic target and presenting the protein in the correct conformation. If the correct antigen is selected and presented to the immune system, then a neutralizing antibody titer may be induced (Van Reeth and Ma, 2013).

Vectored vaccines involve using molecular techniques to cut viral genes out of IAV and then implement them into another virus which is then used as the vaccine platform (Sandbulte et al., 2015). Vectors are rendered non-replicative or are already non-pathogenic in the swine host (Chen et al., 2012). The benefits of this method include induction of both the humoral and cellular immune response, the creation of multivalent vaccines using recombinant technology, and the potential to overcome maternal antibodies (Chen et al., 2012; Ma and Richt, 2010; Vander Veen et al., 2012). One of the complications of the vectored vaccine method is host immunity to the vector. Immunity can be preexisting, develop over time/vaccination, or be maternally derived (Chen et al., 2012). Another complication is the difficulty in making and maintaining a stable vector.

One issue for many vaccines is maternal antibodies, which can be passed from the mother to the offspring through the colostrum (Vincent et al., 2016). Maternal antibodies help protect young swine from natural infection but hinder attempts to prime the immune system since the maternal antibodies inactivate the vaccine antigen prior to inducing an immune response (Chen et al., 2012). This results in naïve swine when the maternal antibodies wane. Naïve swine are susceptible to any strain of IAV that are endemic to the farm (Thacker and Janke, 2008). This is undesirable because it leads to sickness in the swine and maintenance of the virus in the herd. An ideal vaccine platform would be able to circumvent interference from maternally derived antibodies and induce a protective immune response in young swine (Van Reeth and Ma, 2013).

Currently, most commercial vaccines for swine in North America are multivalent inactivated virus vaccines (Loving et al., 2013; Vincent et al., 2016). Multivalent vaccines are usually necessary to induce broad protection since there are many genetic variants of IAV in swine. For example, the human seasonal influenza vaccine is typically a trivalent inactivated whole virus vaccine (Loving et al., 2013). The use of a multivalent vaccine increases the likelihood of protection, but the strains and the adjuvant used also play a role (Takemae et al., 2013). If the vaccine strains do not match the current endemic viruses in a herd, the vaccine is unlikely to induce cross-protection. Likewise, if a poor adjuvant is used, the immune response will not be engaged and antibodies will not develop (Takemae et al., 2013).

Vaccination is recommended for commercial swine, commercial birds, and humans. Influenza viruses can be transmitted, at least transiently, between all three species therefore the best control method is to prevent infection in any of the species (Thacker and Janke, 2008). In particular, it is recommended for animal caretakers to get routine human influenza vaccinations.



It is also recommended that swine herds and turkey flocks be vaccinated to prevent infection from sources of wild-type IAV (Thacker and Janke, 2008).

### **IAV Serological Overview in Swine**

A recent meta-analysis of the swine influenza literature was performed and determined the average seroprevalence of IAV to be about 34.3% in US swine, which varied by year and location (Baudon et al., 2017). The analysis included 217 papers which represented countries from all continents and spanned from 1990 to the present, but only about 30 manuscripts represented the US (Baudon et al., 2017). This suggests there is a limited amount of serological investigation being performed and/or reported, which is not surprising given the difficulties of serological diagnostics. The first major hurdle is determining when animals are sick and need testing. Clinical signs are not always evident thus clinical observation is not always reliable (Detmer et al., 2013; Van Reeth et al., 1996). The second hurdle is the difficulty of obtaining serum samples, which requires a blood draw. This requires trained personnel to physically interact with the animals which can be problematic and time consuming (Detmer et al., 2013). The third hurdle is the difficulty of interpreting serological results. ELISA and HI assays can tell you important information about the animals tested, but only if the results are interpreted correctly, which is becoming more difficult as influenza becomes more genetically and antigenically diverse and issues with cross reactivity, or lack thereof, increase (Detmer et al., 2013; Vincent et al., 2010). A fourth hurdle is producer non-compliance due to the perceived negatives of being discovered as IAV positive and the subsequent economic loss (Detmer et al., 2013). Despite all these hurdles, a number of serological studies have been performed and the utility of serological assays is still important to this day.

*North American IAV Serological Overview*

IAV has been present in US swine herds for several decades and serological reports are periodically performed to evaluate seroprevalence (Baudon et al., 2017). Historically, the H1 has been the dominant subtype of IAV in US swineherds. According to a report from the Minnesota Veterinary Diagnostic Lab (MVDL), which analyzed 111,418 serum over three years, approximately 28.3% of swine serum from 1998 was positive for IAV antibodies and 100% of the antibody positive samples contained antibody against the H1 subtype (Choi et al., 2002b). In 1999, 25.4% of serum tested at the MVDL was positive for IAV antibodies with 74.8% positive for the H1 subtype and 25.2% positive for the H3 subtype (Choi et al., 2002b). By 2001, 20.7% of swine serum tested positive for IAV antibodies with 52.6% of the positive serum samples reacting to the H1 subtype and 47.4% of the positive serum samples reacting to the H3 subtype (Choi et al., 2002b). Changes in seroprevalence could be due to both natural infection and increased vaccination (Choi et al., 2002b). Also it should be noted that these samples came from a veterinary diagnostic laboratory, thus the data generated may be biased since samples are often submitted because infection is suspected within a production system. Also these samples are voluntarily submitted and not actively collected from the field, thus the results may not represent the actual conditions in the field at the time this study occurred.

A recent study surveyed the presence of IAV in swine from the Midwest US during the years of 2009, 2010, and 2011 (Corzo et al., 2013). The study reported that of the 16,170 samples collected, 746 (4.6%) were positive for IAV, which corresponded to 90.6% of farms testing positive for IAV (Corzo et al., 2013). Although the percentage of individual animals positive for IAV may appear low, the percentage of IAV positive farms is still quite high. One

explanation is that the increased rates of vaccination, better biosecurity, and altered management practices have reduced rates of infection of individual animals but has failed to eliminate IAV from farms completely, which persists as subclinical infections (Corzo et al., 2013). The same study also found that H1N1, H1N2, and H3N2 were still the predominant subtypes in US swine. The study also found that mixed infections with two or more IAV subtypes present on a farm were common (Corzo et al., 2013). This study was an active surveillance study and took samples from the field, but the caveat is that the study focused on grower-finisher production swine and not other subpopulations such as replacement gilts and sows on the breeding farm.

Many recent US swine IAV studies, such as the one discussed above, focus on studying the IAV itself and how it changes over time. Often these studies use nasal swabs as the sample type and RT-PCR as the diagnostic test to determine whether samples are positive (Bliss et al., 2016; Diaz et al., 2015; Kaplan et al., 2015). Nasal swabs and RT-PCR based diagnostics are useful because nasal swabs are easier to collect than serum samples and RT-PCR is high throughput and less labor intensive than most serological assays. As a result, serological studies are not as common in the US for commercial swine production systems.

Canadian swineherds have also had to deal with IAV for many years. A serological study evaluating swine from 2001 and 2003 in Ontario using subtype specific ELISAs found that the seroprevalence of H1 subtype antibodies was 61.1% and 24.3% for sows and finishers, respectively (Poljak et al., 2008a). The seroprevalence for the H3 subtype was 0.6% and 0.7% in sows and finishers, respectively (Poljak et al., 2008a). A follow up study performed by the same authors looked at the seroprevalence during 2004 and 2005. The H1 subtype seroprevalence was 13.4% and 14.9% for 2004 and 2005, respectively (Poljak et al., 2008b). The H3 subtype seroprevalence was 2.7% and 25.9% for 2004 and 2005, respectively (Poljak et al., 2008b). A

more recent study by the same authors looked at the seroprevalence of diagnostic cases submitted to the University of Guelph Animal Health Laboratory between 2007 and 2012 (Poljak et al., 2014). The authors evaluated the samples using subtype specific ELISAs and found that 59.6%, 63.9%, and 48.8% of submissions were positive for the H1 subtype, H3 subtype, or both subtypes, respectively (Poljak et al., 2014). These studies show that IAV has been prevalent in Canadian swineherds. These were well thought out studies that utilized both ELISA and HI assays to test samples, collected samples from both finisher swine and sows, and also tried to account for the location and source of the animals involved. The only caveat to these studies is that they did not specifically try to test vaccine antigens when evaluating the antibody responses in the serum samples from the swine they tested.

IAV has also been a problem for swine populations in Mexico (López-Robles et al., 2014; Saavedra-Montañez et al., 2013). Mexican swine production is divided between large intensive production systems, medium sized semi-intensive production systems and small backyard production systems. The IAV viral ecology of Mexico is similar to the US; both countries are dominated primarily by classical swine H1 subtypes, but have had repeated human spillover strains such as human origin H3N2 and H1pdm09. A recent study, which tested over 2000 serum samples from central Mexico over ten years from 2000-2009, found that 74% of the samples were positive for swine H1 subtypes, 24.2% were positive for human H3 subtypes, and 17.8% were positive for H1pdm09 subtypes by the HI assay (Saavedra-Montañez et al., 2013). Another study, testing 150 serum samples from Northwestern Mexico from 2008-2009, found that 55% of the serum was positive for the H1 subtype and 59% of the serum were positive for the H3 subtype via ELISA analysis (López-Robles et al., 2014). These reports suggest a trend where the H1 and H3 subtypes are developing similar prevalence in Mexico. Both studies were

also cross-sectional and thus addressed seroprevalence within all swine subpopulations, although they focused on production farms and not breeding farms.

### **Conclusions and Objectives**

IAV is an important respiratory pathogen of swine. The virus causes respiratory illness in swine around the world. In the US, the viral diversity has been increasing over the last two decades. The introduction of the 1998 human like H3N2 and the rise of the 2009 pandemic H1N1 have both resulted in an explosion of viral genetic diversity. This diversity has caused difficulties for vaccine development as it has become difficult to create a vaccine to protect against all the strains of IAV present in US swineherds. Over the years, there has also been a decrease in serological surveillance of swineherds, which can help determine levels of protection. Replacement gilts in particular are a neglected subpopulation in discussion about IAV ecology. To help fill this gap, the following study was performed in new replacement gilts to evaluate the IAV antibody status in pre- and post-vaccination gilts. This study sought to evaluate homologous vaccine antibody responses and potential cross reactive antibodies in vaccinated gilts.

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**CHAPTER 3. EVALUATION OF PRE-VACCINATION AND POST-VACCINATION  
INFLUENZA A VIRUS ANTIBODY RESPONSES IN BREEDING AGE  
REPLACEMENT GILTS FROM BREEDING FARMS IN THE UNITED STATES**

*Modified from a paper to be published in the Journal of Veterinary Microbiology*

Running title: Influenza A virus antibody in replacement gilts.

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**Abstract**

The genetic diversity of influenza A virus (IAV) circulating in swine has been increasing over the previous twenty years. Surveillance of gilts for IAV pre- and post-vaccination antibody status are lacking at the farm level which may impact the health of breeding herds. This study sought to evaluate the IAV antibody response in breeding age, new replacement gilts prior to vaccination and to assess the IAV vaccine antibody response after integration into the herd. This study included 1,293 serum samples (504 pre-vaccination samples and 789 post-vaccination samples) that were collected from 12 breeding farms throughout the United States for approximately one year. Samples were analyzed by nucleoprotein (NP) enzyme-linked immunosorbent assay (ELISA) and hemagglutination inhibition (HI) assay using farm-specific homologous vaccine antigens and six IAV representing the predominant genetic and antigenic clusters circulating in swine. Approximately 38.3% (193/504) of the gilts, representing 50% (6/12) of the farms, were NP ELISA antibody positive prior to IAV vaccination (Pre-Vac) and entry into the breeding farm. Collectively, five farms lacking Pre-Vac antibodies demonstrated a more uniform homologous IAV vaccine antibody response. Influenza vaccines failed to induce HI antibodies in 100% of gilts from nine farms after receiving 4 doses of vaccine. The NP ELISA and HI antibodies detected on three farms using a subunit HA vaccine suggested natural IAV exposure occurred prior to vaccination. In summary, the IAV antibody status of replacement gilts can be monitored using serological assays pre- and post-vaccination which may help develop more effective vaccination strategies.

## Introduction

Influenza A virus (IAV) is a negative sense, single stranded, enveloped, RNA virus of the *Orthomyxoviridae* family and a cause of respiratory disease in mammalian species including swine (Vincent et al., 2008). The three predominant IAV subtypes in swine that currently co-circulate in the United States (US) are H1N1, H1N2, and H3N2 (Nelson et al., 2015b). IAV is endemic in US swine causing high morbidity but low mortality (Vincent et al., 2008). Influenza A virus may also result in significant economic losses to US swine producers due to decreased weight gain, plus increased treatment and vaccination costs (Haden et al., 2012).

IAV in swine are placed into multiple different phylogenetic clades based on nucleotide differences in the two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) (Van Reeth et al., 2012). Currently, there are sixteen IAV-S phylogenetic clades in the US. Eight of the genetic clades are of the H1 subtype ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\gamma$ 2,  $\delta$ -1a,  $\delta$ -1b,  $\delta$ -2, and pandemic 2009 H1N1) and eight additional genetic clades are of the H3 subtype (IV, IV-A, IV-B, IV-C, IV-D, IV-E, IV-F, and 2010 human-like H3) (Lewis et al., 2016; Vincent et al., 2014). The antigenic properties of the different genetic clades within a subtype are diverse and antibody cross reactivity is difficult to determine without appropriate serological assays.

Increasing IAV-S genetic diversity has made serological surveillance and vaccination more difficult (Goodell et al., 2016; Sandbulte et al., 2015). Increased IAV antigenic diversity has complicated the interpretation of serological assays, such as the nucleoprotein enzyme-linked immunosorbent assay (NP ELISA) and hemagglutination inhibition (HI) assay, due to decreased cross reactivity and increased chances of a mismatch between test antigen and serum antibodies (Goodell et al., 2016; Rajao et al., 2014). Increased IAV antigenic diversity has decreased the ability of vaccines to broadly cross-protect against different strains of the virus and updating



vaccines with relevant antigens has also become more difficult (Vincent et al., 2017). Due to the potential for mismatch between vaccine antigen and endemic viral strains, IAV-S vaccines need to be constantly evaluated and updated (Sandbulte et al., 2015). Serological tests, such as the HI assay, are best used for farm specific diagnostic questions such as evaluating IAV-S vaccine antibody responses when the previous IAV history of the farm is well-known.

Swine farms in the US represent dynamic production systems with individual flows of breeding, gestation, farrowing, nursery, grow-finish, and wean to finish farms (Knauer and Hostetler, 2013). Each flow or subpopulation within a farm may represent different ecological niches of IAV-S affecting the introduction or maintenance of new or endemic virus (Diaz et al., 2017b). One important subpopulation is replacement gilts which may replace approximately 50% of the adult breeding herd per year (Penmetchsa et al., 2009). Therefore, new replacement gilts can impact breeding herd health in a short period of time.

The objectives of this study were twofold. The first objective was to characterize the IAV antibody status of non-vaccinated, breeding-age replacement gilts prior to entering the breeding farm. The second objective was to evaluate the post-vaccination, homologous vaccine antibody responses in new replacement gilts over their first year of residence in the breeding farm. The NP ELISA and HI assay were used to evaluate the pre- and post-vaccination antibody response during four time points over one year. Additionally, six IAV-S representing the predominant genetic and antigenic clusters circulating in swine were used in HI assays to evaluate the potential presence of pre- and post-vaccination cross reactive antibodies. Monitoring the IAV antibody status of new replacement gilts may justify the need to update vaccine antigens or adjust vaccination protocols to more effectively control IAV-S in breeding farms.

## Materials and Methods

### *Experimental Design*

Twelve commercial breeding swine production systems (breeding farms) from eleven different states in the US were actively recruited for this IAV antibody study. Three farms were selected from each of the regions 1-4 of the United States Department of Agriculture (USDA) IAV-S surveillance program (USDA, 2017). The target population was non-vaccinated new replacement gilts prior to entry into the main breeding farm. For each farm, a minimum of 40 serum samples were requested for the pre-vaccination (Pre-Vac) time period. The serum samples were randomly collected by local veterinarians and/or farm personnel. Samples were submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL), processed via centrifugation at 800x *g* if needed, and maintained at -80°C prior to analysis.

During the post-vaccination time periods, a subset of 20 replacement gilts were randomly selected for serum collection from the initial 40 gilts. A minimum of 20 serum samples were requested; however, gilts may have been removed due to illness, lameness, trauma, or other unknown reasons thus reducing the number of samples below the target. Samples were collected by the veterinarian or farm personnel and the same cohort of animals were sampled at each time point. Samples were sent to the ISU VDL, processed by centrifugation if needed, and frozen at -80°C to await analysis. The gilts were bled for a series of three time periods representing Post-Vaccination (Post-Vac), Post Integration 1 (P-Int 1), and Post-Integration 2 (P-Int 2). It should be noted the Oklahoma (OK) herd was removed from the study due to a PRRS outbreak after the Pre-Vac time period. The experimental design is described in Table 1.

### *Farm Questionnaire*

A questionnaire was sent to the farm veterinarians to gather gilt multiplier and breeding farm demographic information. Approximately eight questions focused on the gilt source and the sow farm demographics and included questions regarding multiplier location, influenza status of the multiplier site, type of multiplier, age at gilt selection, sow farm location, number of breeding animals, and number of replacement gilts. A second set of eight questions focused on vaccination protocols including vaccine platform, commercial or farm specific vaccine, number of prime and boost doses, timing of vaccination, isolation protocols, and gilt age at integration into the breeding herd.

### *Influenza A Virus Antibody Assays*

Serum samples were evaluated using a nucleoprotein enzyme-linked immunosorbent assay (NP ELISA) (Swine Influenza Virus Ab Test, IDEXX Laboratories, Inc., Westbrook, Maine) per manufacturer's instructions. Briefly, serum was diluted with Dilution Solution from the kit to a 1:10 prior to testing and 100 µl of diluted serum were added to IAV NP-coated plates, incubated at room temperature for 60 minutes, washed three times with 300 µl of wash solution, followed by 100 µl of anti-NP conjugate, incubation at room temperature for 30 minutes, followed by 100 µl of TBM substrate, incubation for 15 minutes at room temp, followed by 100 µl of stop solution. Color development was evaluated with an ELISA plate reader (Molecular Devices Corp., Sunnyvale, CA) at a wavelength of 650 nm to determine optical density (OD). One replicate per sample was used while two replicates of the positive control (PC) and negative control (NC) were included with each set of samples tested. To determine the validity of the assay, the average OD of PC and NC were compared with an average NC value of  $\geq 0.60$  and a

PC/NC value of  $<0.50$ . Results were reported as a sample to negative (S/N) ratio by dividing sample OD by the average of the NC negative OD. Per manufacturer instructions, an S/N ratio  $\leq 0.60$  was considered positive for IAV-S NP-ELISA antibody. An S/N ratio  $>0.60$  was considered negative for IAV-S NP-ELISA antibody (Goodell et al., 2016).

Sera were also evaluated using a HI assay based on a previous protocol with modifications (Kitikoon et al., 2014). Prior to testing, sera were treated with receptor-destroying enzyme (RDE) (Denka Seiken., Japan) overnight at  $37^{\circ}\text{C}$  at a mixture of 1 volume serum to 4 volumes RDE to remove non-specific inhibitors of agglutination. The following day, 5 volumes of 0.85% sterile saline was added to the mixture followed by heat inactivation at  $56^{\circ}\text{C}$  for 60 minutes. Next, sera were adsorbed with 0.5 volumes of 50% turkey red blood cells (RBCs) for one hour, after which the serum was centrifuged at  $800\times g$  to remove the RBCs and transferred to a new plate. The RBC adsorption procedure was performed twice to remove natural serum agglutinins. Post treatment, sera were at a final dilution of 1:10 and used in HI assays with 0.5% turkey RBC's as the indicator and virus antigen was diluted to 8 hemagglutination units (HAU). 25  $\mu\text{l}$  of sera were serially two-fold diluted in a V-bottom plate (Thermo Fischer Scientific, Rochester, New York) containing 25  $\mu\text{l}$  of phosphate buffered saline (PBS) (Gibco, Grand Island, New York). Virus antigen at 8 HAU in 25  $\mu\text{l}$  was added to each dilution, incubated for 30 minutes at room temperature, followed by the addition of 50  $\mu\text{l}$  of 0.5% RBCs. After a 30 minute incubation, results were reported as the highest serum dilution that inhibited RBC agglutination. An HI titer  $\geq 40$  was considered positive, HI titers = 20 were considered suspect, and HI titers  $\leq 10$  were considered negative. The percent HI antibody positive gilts were calculated for each antigen on each on farm at each time period.

### *Virus Antigens*

Influenza A virus antigens in the HI assay were acquired from the ISU VDL or the United States Department of Agriculture National Veterinary Services Laboratory (USDA NVSL). The IAV-S were propagated in Madin-Darby Canine Kidney (MDCK) cells (ATCC CCL-34), clarified through centrifugation at 800x g, and analyzed for HAU as previously described (Kitikoon et al., 2014). Virus stocks were aliquoted in 4 ml amounts, stored at -80° C, and then diluted to 8 HAU prior to testing.

The USDA IAV surveillance data was used to select the six viruses representing the major genetic clades and antigenic clusters currently circulating in US swine during the 2015 season (Abente et al., 2016; Anderson et al., 2015; Anderson et al., 2013). Three H1 and three H3 were chosen to evaluate the presence of cross reactive HI antibodies in the serum using the HI assay. The six representative antigens are further described in Table 3.

Sera were also evaluated in HI assays using commercial or farm-specific antigens used in IAV vaccines in their respective breeding farms. When possible, commercial or farm specific virus antigens or isolates were acquired from the ISU VDL or USDA NVSL, propagated in MDCK, clarified by centrifugation prior to use in the HI assay. Putative antigens were used when commercial vaccine or farm-specific vaccine IAV isolates were not available. Putative IAV antigens were chosen based on H1 amino acid sequence homology (>99.0%) with the HA1 portion of the genome (first 200 bp of the HA) and similarity with H1 HA putative neutralizing epitopes, six H3 HA antigenic sites (amino acid positions 145, 155, 156, 158, 159, and 189), or IAV serological cross-reactivity (Abente et al., 2016; Caton et al., 1982). For the FluSure XP (Zoetis, Parsippany, NJ) antigens, virus isolates were unavailable and instead purified HA

proteins were provided by the ISU VDL and included H1- $\gamma$ , H1- $\delta$ 1, H1- $\delta$ 2, and H3-IV. Putative vaccine antigens were chosen for MaxiVac Excell 5.0 (Merck Animal Health, De Soto, KS) based on IAV serological cross-reactivity to vaccine-specific antiserum. MaxiVac Excell 5.0 putative vaccine antigens included A/Swine/Kentucky/02086/2008(H1- $\beta$ ), A/Swine/Ohio/02026/2008(H1- $\gamma$ ), A/Swine/Iowa/02955/2010(H1- $\delta$ 2), and A/Swine/Minnesota/02782/2009(H3-IV). Maxi Vac putative antigens are further described in Table 3.

## Results

### *Breeding Farm and Gilt Demographics Overview*

The number of serum samples collected at each time period is reported in Table 1. The age of replacement gilts at selection or arrival to the breeding farm isolation or gilt developer unit (GDU) averaged twenty-four weeks old but varied based on the type of multiplication system (internal or external). Production systems utilizing internal multiplication selected gilts at a younger age than production systems that externally purchased gilts at breeding age (28-30 weeks). Replacement gilts were sourced from an off-site location in 75% (9/12) of the sow farms with 44.4% (4/9) of those farms receiving replacement gilts from North Dakota (IA, IN, PA, ND). Approximately 41.7% (5/12) of the sow farms also reported sourcing their replacement gilts from known or perceived influenza negative sites (IA, IN, ND, PA, NC-1). Farm demographics are summarized in Table 2.

Ten of the eleven remaining farms enrolled in the study vaccinated their replacement gilts. Commercial IAV vaccines were administered in 60% (6/10) of the farms. Five farms (MO, NC-1, NC-2, IN, IL) used Zoetis FluSure XP<sup>®</sup> and one farm (TX) used MaxiVac Excell<sup>™</sup> 5.0.

Farm-specific autogenous or custom vaccines were used in 50% (5/10) of the farms. Of the farm-specific vaccines, NE used a bivalent vaccine, and IA and IL used a trivalent vaccine. KS used a quadrivalent vaccine and PA used a pentavalent vaccine. Four of the farms (IA, KS, NE, IL) used an HA subunit vaccine based on the replicon particle (RP) technology (Merck Animal Health, Madison, NJ). The IL farm switched from a commercial product to a subunit replicon particle vaccine (trivalent) between the P-Int 1 and P-Int 2 sample collections. The ND farm did not vaccinate their breeding herd.

Isolation protocols were used in only 36.4% (4/11) of the farms, ranging from a minimum of four weeks up to ten weeks, whereas 63.6% (7/11) farms did not have a specific isolation protocol for replacement gilts. Average gilt age at herd entry was approximately 30 weeks. Vaccine was administered in two doses prior to herd entry in 80% (8/10) of the farms. One farm, NE, administered only one dose of vaccine prior to herd entry and one farm, PA, administered two doses of vaccine after gilts were placed into the main breeding herd. The primary dose of IAV vaccine was administered approximately -10 to -4 weeks prior to placing gilts into the main breeding herd with the booster dose ranging from -7 to 0 weeks prior to entry. Time between doses, where applicable, averaged approximately 3 weeks. Farm demographic information can be found in Table 2.

### *Pre-Vac Overview*

Influenza NP ELISA antibodies were detected in 38.3% (193/504) of the gilts and 50% (6/12) of the farms had at least one NP ELISA positive replacement gilt. The NP ELISA negative farms included IA, IN, IL, NC-1, NC-2, and OK. The farms receiving NP ELISA positive gilts included KS, MO, ND, NE, PA, and TX. Two farms presumed to be receiving IAV

negative gilts, PA and ND, had 25.6% and 65.0% of the gilts test NP ELISA antibody positive, respectively. Conversely, NC-2, IL and OK farms presumed receiving IAV positive gilts did not demonstrate NP ELISA antibody positive gilts. Four of six farms with NP ELISA positive gilts reported internal multiplication, in contrast to four of six farms with NP ELISA negative gilts that reported external multiplication. Approximately 83.3% (5/6) of the farms receiving NP ELISA positive gilts had greater than 50% of the gilts positive for NP ELISA antibody prior to first vaccination.

Influenza HI assay antibodies to one or more representative antigens were detected in 16.9% (85/504) of the gilts and 41.7% (5/12) farms. The farms receiving HI antibody positive gilts at the Pre-Vac time period included KS, MO, ND, NE, and TX. The KS and TX farms had 62.5% (25/40) gilts test HI antibody positive to the H1- $\gamma$  and 74.0% (37/50) gilts test HI antibody positive to the H1- $\delta$ 1 representative antigens, respectively. The farms receiving HI antibody negative gilts to the representative antigens at the Pre-Vac time period included IA, IL, IN, NC-1, NC-2, OK, and PA.

#### *Post-Vac Overview*

NP ELISA antibody positive gilts were detected in 100% (10/10) of the vaccinated farms at Post-Vac, P-Int 1, and P-Int 2 suggesting natural exposure occurred in breeding farms using the subunit HA vaccine. The IN and NC-2 farms had 100% of replacement gilts test NP ELISA antibody positive at all three time periods; both farms used the FluSure commercial vaccine. The IA and KS farms did not have a single time period where 100% of replacement gilts tested NP ELISA antibody positive; however, both farms used subunit replicon particle vaccines. The percent NP ELISA positive replacement gilts ranged from 77.3% to 100% at Post-Vac, 38.1% to



100% at P-Int 1, and 81.8% to 100% at P-Int 2 due to vaccination or natural exposure. The percent NP ELISA antibody positive replacement gilts varied within and across farms and time periods.

The percent of gilts with positive homologous HI antibodies varied widely from 0.0% to 100%, across all farms and based on vaccine type, individual vaccine antigen, and time period. Percent HI antibody positive Post-Vac gilts from farms using a commercial IAV vaccine ranged from 0% to 94.9%. The P-Int 1 and P-Int 2 percent HI antibody positive gilts in farms using commercial vaccines ranged from 4.3% to 100% and 18.2% to 100%, respectively. Four of five farms (NC-1, NC-2, IL, IN) still using commercial IAV vaccines at P-Int 2 had 100% of the gilts HI antibody positive to at least one antigen. However, only one farm (NC-2) at P-Int 2 had 100% HI antibody positive gilts to all four FluSure antigens. Most farms had at least one of the commercial vaccine antigens at P-Vac, P-Int 1, and P-Int 2 that did not induce an HI antibody response in one or more gilts. Two farms, TX and MO, had <10% of gilts demonstrate a positive HI antibody titer to three of their vaccine antigens after two doses of vaccine at the P-Vac time point. Percent positive gilts with HI antibody titers remained low, 4.3% to 54.5%, for the H1 vaccine antigens throughout all time periods in the MO farm.

Breeding herds using farm specific IAV antigens demonstrated a similar level of variability in the percent positive replacement gilts within and across farms. The percent positive gilts with HI antibody titers from herds using farm specific antigens ranged from 0.0% to 85.3% at P-Vac, 0.0% to 85.7% at P-Int 1, and 0.0% to 100% at P-Int 2 across all antigens. The four farms that used a farm specific IAV vaccine had at least one or more antigens that were unable to induce a positive HI antibody titer in >50% of the gilts at P-Vac after receiving two doses of vaccine, including the NE gilts although they received only one dose of vaccine. Three farms had

at least one antigen in their farm specific vaccine that failed to induce a positive HI antibody titer in at least 50% of the gilts at P-Vac, P-Int 1, or P-Int 2. The KS farm had one antigen (2014 H1- $\delta$ 2) that was unable to detect a positive HI antibody response in any of the gilts across all time points.

The percent HI antibody positive gilts to the six representative antigens was highly variable post vaccination and obvious trends were not observed. Percent positive gilts ranged from 0% to 100% based on representative antigen, farm, and time period. Cross reactivity was difficult to predict as some farms displayed HI antibody positive gilts to representative antigens that were of the same phylogenetic cluster as the vaccine antigens while some farms did not. In addition, 7 of the 10 vaccinated farms displayed >50% of gilts testing positive to a representative antigen from a cluster that was not included in the vaccine. An example is the IA farm which displayed >60.0% HI antibody positive gilts to the H3-Green representative antigen at Post-Vac, P-Int 1, and P-Int 2, despite the vaccine administered not containing an H3-Green (H3-IVA) antigen. Since each farm presented different pre- and post-antibody statuses and there were no general trends among all farms, we next evaluated the responses individually by farm.

*Gilts without pre-vaccination IAV antibodies responded more uniformly to vaccine*

The NC-2 farm is an example of how serologically negative gilts can have a more uniform IAV vaccine response. The NC-2 farm used the FluSure commercial product, externally purchased their replacement gilts from Mississippi, and reported that their multiplier source was IAV positive (Table 2). Reviewing the Pre-Vac data, none of the new replacement gilts from the NC-2 farm tested NP ELISA antibody positive at the Pre-Vac time period although the status of the multiplier may have been IAV positive (Figure 1A). The NC-2 gilts also tested HI antibody

negative to all six of the representative antigens in the HI Assay (Figure 1B). These results suggest a lack of recent exposure in these gilts and/or waning of maternal antibodies below the level of detection by the assay.

Naïve gilts from the NC-2 farm administered the commercial FluSure vaccine demonstrated an adequate Post-Vac HI antibody response, overall. The replacement gilts received two doses of vaccine prior to serum collection at Post-Vac. Testing with homologous HI vaccine antigens demonstrated >69.0% of the gilts HI antibody positive to three of the four vaccine antigens (Figure 1A). The fourth antigen had a reduced number of positive gilts at 27.8%. After a third dose of vaccine at the P-Int 1 time period, >96.0% of the replacement gilts were HI antibody positive to all four vaccine antigens. The percentage of HI antibody positive gilts increased to 100% for all antigens by the P-Int 2 time period. This suggests that 3 doses of FluSure induced a more uniform HI antibody response in naïve gilts based on the vaccination protocol specific to the farm.

The NC-2 farm displayed a number of gilts with cross reactive antibodies to the representative virus antigens. At Post-Vac, >50.0% of the gilts tested positive to both the H1- $\delta$ 1 and H1- $\delta$ 2 representative antigens. By P-Int 2, >95.0% of gilts tested HI antibody positive to the H1- $\gamma$ , H1- $\delta$ 1, and H1- $\delta$ 2 representative antigens (Figure 1B). This is was expected since the FluSure vaccine contains H1- $\gamma$ , H1- $\delta$ 1, and H1- $\delta$ 2 vaccine antigens suggesting the representative antigens may have been antigenically similar to the vaccine strains. Additionally,  $\geq$ 50.0% of the gilts were HI antibody positive to the H3-Red representative antigen, starting in the Post-Vac time period, and the H3-Green representative antigen, starting in the P-Int 1 time period. This is could represent a potential novel IAV infection since the antigenic properties of the FluSure vaccine H3 component are unknown.

*NP ELISA positive gilts at vaccination demonstrated reduced HI antibody responses to vaccine antigens*

The MO farm is an example of how IAV antibody positive gilts may respond immunologically to inactivated, whole virus IAV vaccination, using the NP ELISA and HI assay. The MO farm also used the FluSure commercial product, externally purchased their gilts from Illinois, and reported their multiplier location was positive for IAV (Table 2). At the Pre-Vac time period, 87.5% of the new replacement gilts tested NP ELISA antibody positive (Figure 1C). This suggests natural IAV exposure or residual passive antibodies within this population of replacement gilts considering gilts were not IAV vaccinated prior to analysis. This was further supported by the detection of cross reactive HI antibodies to one or more of the six representative antigens. Specifically, 22.5% and 12.5% of the gilts tested HI antibody positive for the H3-Green and H3-human like representative antigens, respectively (Figure 1D).

The MO farm gave two doses of the FluSure vaccine prior to serum collection for the Post-Vac time period. In sharp contrast to the NC-2 antibody negative herd, none of the gilts tested HI antibody positive to the three H1 vaccine antigens, suggesting vulnerability to an H1 IAV infection during this time period. The percentage of positive gilts to the H1 vaccine components increased at the P-Int 1 time period after receiving a third dose, with 21.7%, 13.0%, and 4.0% of the gilts testing HI antibody positive for the H1- $\gamma$ , H1- $\delta$ 2, and H1- $\delta$ 1 vaccine antigens, respectively. By the P-Int 2 time period the percentage of HI antibody positive gilts for the H1 subtype antigens were only 45.5%, 54.5%, and 18.2% for the H1- $\gamma$ , H1- $\delta$ 2, and H1- $\delta$ 1 vaccine antigens, respectively. The H3 response was impacted to a lesser degree, as 65.6% of the

gilts tested HI antibody positive to the H3-IV vaccine antigen at the Post-Vac time period and increased to 78.3% and to 81.8% at P-Int 1 and P-Int 2, respectively.

The MO farm had low levels of cross reactive antibodies to the six representative antigens. With the exception of the H3-Green and H3-human like representative antigens, cross reactive antibodies were <37% for all antigens at all time periods (Figure 1D). At P-Int 1, 78.3% and 60.9% of gilts on the MO farm tested HI antibody positive to the H3-Green and H3-human like representative antigens. By P-Int 2, 95.5% of gilts tested HI antibody positive to the H3-Green representative antigen, which could be due an ongoing novel infection with an H3-Green virus that does not cross react with the FluSure H3 component. The results are unclear as the antigenic properties of the FluSure H3 component are unknown.

*Post vaccination NP ELISA positive gilts suggests natural exposure on farms using an HA subunit vaccine*

The IA farm was an example of a farm where IAV natural infection likely occurred based on the NP ELISA and HI assay data. The IA farm used a trivalent, farm specific subunit HA replicon particle vaccine, purchased their replacement gilts from an external source in North Dakota, and reported the multiplier location was IAV negative (Table 2). The vaccine contained an H1- $\gamma$ , H1- $\delta$ 1, and H3-human like HA RNA components (Table 3). Since the replicon particle vaccine did not include an NP RNA component, an NP antibody response post vaccination is suggestive of natural exposure. None of the replacement gilts from the IA farm tested NP ELISA antibody positive at the Pre-Vac time period, yet >97.1% of gilts tested NP ELISA antibody positive at the Post-Vac time period following integration into the breeding herd (Figure 2A). The percentage of NP ELISA antibody positive gilts remained high throughout the time periods

with 74.3% and 86.5% of gilts NP ELISA antibody positive at the P-Int 1 and P-Int 2 time periods, respectively.

The vaccine antibody response was variable between the different antigens within the vaccine, as measured by the percent HI antibody positive gilts. The H1- $\delta$ 1 antigen had the most robust response with >85.0% of the replacement gilts testing HI antibody positive at all time periods. The H1- $\gamma$  antigen had a lower response with only 26.5% of gilts testing HI antibody positive at Post-Vac and rising to 67.6% of gilts testing HI antibody positive at P-Int 2. The H3-human like antigen had a poor response with <28% of gilts testing HI antibody positive at all time periods. While the vaccine antibody response was variable by antigen, antibody responses to all antigens increased over time.

The NP ELISA antibodies suggest natural infection, and a large percentage of the replacement gilts from the IA farm had cross reactive antibodies to the representative antigens. The vaccine used did not contain an H3-Green (H3-IVA) antigen, yet 61.8% of the replacement gilts tested HI antibody positive to the H3-Green representative antigen at the Post-Vac time period (Figure 2B). The percentage of HI antibody positive gilts to the H3-Green representative antigen continued to increase to 82.9% and 97.3% for the P-Int 1 and P-Int 2 time periods, respectively. There was also evidence of natural infection due to an H1- $\delta$ 2 strain of IAV. At the P-Int 2 time period, 100% of replacement gilts tested HI antibody positive to the H1- $\delta$ 2 representative antigen (Figure 2B). Less than 3% of the gilts tested HI antibody positive to the H1- $\delta$ 2 representative antigen prior to the P-Int 2 time period.

*NP ELISA and HI antibodies waned without further boosting in a negative sow herd*

The North Dakota farm reported that they did not vaccinate their new replacement gilts, internally produced their gilts, and that their multiplication site was influenza negative. However, the NP ELISA and HI assay results indicate the gilts were exposed prior to entering the breeding herd. At the Pre-Vac time period, 65.0% of the replacement gilts tested NP ELISA antibody positive and 22.5% of replacement gilts tested HI antibody positive to the H3-human like representative antigen (Figure 2C). This suggests possible IAV exposure in these gilts despite the farm presumption that the multiplier site was IAV negative. The antibody assays cannot rule out human seasonal H3 exposure or exposure to the 2010 hu-like H3N2 currently circulating in the US swine population (Rajão et al., 2015). The observed Pre-Vac antibody responses waned by the Post-Vac time period, suggesting that IAV was circulating at a low prevalence or not at all in the main breeding herd and additional exposure to human-seasonal H3 was lacking. All replacement gilts were NP ELISA antibody negative at Post-Vac, P-Int 1, and P-Int 2. Also, all replacement gilts were HI antibody negative for cross reactive antibodies to the H3-human like representative antigen at Post-Vac, P-Int 1, and P-Int 2. These data suggest that IAV antibody wanes quickly unless boosted by natural infection or vaccination and that negative sow farms may be at risk for human seasonal or swine-origin IAV infection.

## **Discussion**

Epidemiological studies of IAV in swine reported in the literature frequently focus on detecting and sequencing virus (Corzo et al., 2013; Diaz et al., 2017a; Diaz et al., 2017b). Serological surveillance has often targeted adult breeding swine or slaughter pigs but few replacement gilts (Kyriakis et al., 2013; Van Reeth et al., 2008). The aim of the current study

was to evaluate the IAV antibody status of non-vaccinated, new replacement gilts prior to vaccination and following entry into a breeding herd. Post vaccination and post entry into a breeding herd IAV antibody responses were evaluated using homologous vaccine antigens and six representative antigens representing dominant IAV circulating in the swine population identified by USDA surveillance. The initial pre-vaccination serum samples were collected at approximately 24 weeks of age, and were evaluated for the presence of IAV antibodies using the NP ELISA and HI assay using six representative antigens. The post vaccination serum samples were collected at three time periods over one year, from a subset of the initial set of gilts, and tested for IAV antibodies using the NP ELISA and HI assay with homologous vaccine antigens and six representative antigens.

Endemic IAV infections are common in breeding herds in the US, although many remain undiagnosed because clinical signs are mild and diagnostic tests, such as serological assays, are not performed routinely (Simon-Grifé et al., 2012; White et al., 2017). In our study, 58.3% (7/12) of the farms enrolled reported sourcing their gilts from IAV positive multiplication sites. It is unclear if the IAV status was based solely on observed clinical disease or diagnostic testing. In either case, it appears diagnostic testing was under-utilized since two farms (ND and PA) reported sourcing their new replacement gilts from an IAV negative multiplier yet >20% of their gilts tested IAV antibody positive. Additionally, three farms (NC-2, IL, and OK) reported sourcing their gilts from IAV positive sites were instead IAV antibody negative at Pre-Vac. This demonstrates the importance of conducting serological testing on new replacement gilts to more accurately determine IAV antibody profiles.

Knowledge of replacement gilt IAV antibody status is important considering pre-existing IAV antibody may influence vaccine antibody responses and there are efforts to maintain IAV



free replacement gilts within the swine industry (Corzo et al., 2014). Vaccinating serologically negative gilts may be preferential to avoid maternal antibody and/or prior immunity interference. In contrast, IAV antibody negative gilts may be vulnerable to natural infection from nearby farms or endemic strains when they are integrated into the breeding farm (Poljak et al., 2008). An example of how IAV antibody negative gilts respond effectively to vaccination was demonstrated in the NC-2 farm, where all gilts were NP ELISA and HI antibody negative at Pre-Vac. Upon vaccination, there were HI antibody responses in >69.0% of the gilts against three of the four vaccine antigens. By the P-Int 2 time period, 100% of the gilts tested were HI antibody positive to all four of the vaccine antigens. Although the ideal response would be 100% positive to all 4 antigens after 2 doses, compared to farms that vaccinated IAV antibody positive gilts, the results from this farm provided evidence that vaccinating serologically negative gilts was beneficial and yielded a stronger antibody response.

The MO farm utilized the same vaccine as the NC-2 farm, but was an example of how pre-existing antibodies may interfere with vaccine antibody responses (Fazekas de St Groth and Webster, 1966; Fonville et al., 2016; Vincent et al., 2017). At the Pre-Vac time period, 87.5% of the replacement gilts tested NP ELISA antibody positive and 22.5% tested HI antibody positive to the H3-Green (H3-IVA) representative antigen. Taken together, the assay results suggest that there was significant IAV exposure prior to the Pre-Vac time period, including but not limited to a virus of the H3-green antigenic cluster. The pre-existing antibodies may have interfered with the vaccine antibody response since none of the replacement gilts tested HI antibody positive for the three H1 subtype vaccine antigens (H1- $\gamma$  H1- $\delta$ 1, and H1- $\delta$ 2) at the Post-Vac time period. The antibody response continued to be muted to the test antigens through the P-Int 2 time period with <55% of the gilts testing HI antibody positive against the H1-subtype vaccine antigens. The MO

farm shows the impact that pre-existing IAV antibodies can have on vaccine antibody responses in replacement gilts.

The IA farm is an example of the utility of the NP ELISA and HI assays used in combination to differentiate natural infection from vaccination. The IA farm used a RP vaccine, which delivers RNA for the HA protein to host cells, thus it should not induce an NP antibody response (Vander Veen et al., 2012). This allows for the NP ELISA to potentially evaluate endemic infection within a breeding herd that uses an RP vaccine. None of the new replacement gilts from the IA herd tested NP ELISA or HI antibody positive at the Pre-Vac time period. However, after entry to the breeding herd and vaccination, 97.1% of replacement gilts tested NP ELISA and HI antibody positive to the H3 hu-like representative antigen as well as 61.8% testing HI antibody positive to the H3-Green representative antigen. These results suggest that a potential natural exposure to an H3-human like and H3-Green (H3-IVA) may have occurred between the Pre-Vac and Post-Vac sample collection time periods. There was also evidence for interference with the hu-like H3 component of the vaccine since only 2.9% of the replacement gilts tested HI antibody positive to the H3-human like vaccine antigen. Potentially, antibody blunting may have occurred due to the two viruses being homosubtypic and antigenically similar and as a result the previous exposure could have prevented the immune system from recognizing the vaccine antigen and thus the immune system did not mount a novel antibody response, but further testing comparing the two viruses genetically and antigenically would need to be performed. At P-Int 2 the percentage of NP ELISA positive replacement gilts was still high at 86.5% of gilts, which was suggestive of continued endemic circulation of IAV within the herd. The HI assay data supports that a second virus of the H3-Red antigenic cluster may have entered the breeding herd at P-Int 1 with 57.1% of gilts testing HI antibody positive and that a H1- $\delta$ 2

may have also entered the breeding herd at P-Int 2 with 100% of the replacement gilts testing HI antibody positive. The IA herd shows how the NP ELISA and HI assay can be used to determine potential endemic viral infections on farms that use a replicon particle vaccine and help producers formulate changes to their vaccine products and vaccination protocol.

The ND farm provided another example of the importance of serological surveillance that combines NP ELISA and HI assays. The ND farm self-reported as maintaining an IAV negative breeding herd and gilt multiplier. The farm is located in a low swine dense area of the US, produce their gilts internally, and do not purchase external gilts. However, 65.0% of the replacement gilts tested NP ELISA antibody positive at Pre-Vac. Additionally, 22.5% of the replacement gilts tested HI antibody positive to the H3-human like representative antigen. This exposure could have been due to either a contemporary human seasonal H3N2 from infected farm workers or to the currently endemic 2010 hu-like H3 swine virus. Human seasonal spillovers into swine are not uncommon, but human viruses often fail to transmit onward to become established in swine (Nelson et al., 2015a; Nelson and Vincent, 2015). This may have been the case as by the Post-Vac time period, as none of the replacement gilts tested NP ELISA antibody positive or HI antibody positive to the H3 hu-like representative antigen. Additionally these data suggest that IAV antibodies quickly waned, within 6-8 weeks, to undetectable levels without a stimulating exposure; either natural infection or vaccination. This data supports the importance of routinely performing diagnostic serologic testing rather than assuming serological status based on previous history.

Collectively, the data from this study demonstrates the importance of serological testing to confirm the status of new replacement gilts entering the breeding farm as IAV negative or with evidence of prior exposure to endemic IAV present at the genetic multiplier. Replacement

gilts that are IAV antibody negative may be advantageous since they are less likely to transmit new viruses to breeding farm and are more likely to have a robust vaccine antibody response to the vaccines used on the breeding farm. However, antibody negative gilts may be vulnerable to exposure to endemic IAV in the main breeding herd if exposure occurs within the first weeks of being on a production site prior to induction of immune responses to inactivated IAV vaccines. Naïve or partially immune incoming gilts may thus help maintain endemic infections within a breeding farm. Increased IAV serological monitoring of replacement gilt populations can help identify exposure history and/or residual passive antibody, therefore help develop new vaccination protocols or improve the timing of existing vaccination protocols to maximize antibody responses, and ultimately determine the best strategy for introducing replacement gilts to the adult breeding herd. Several of the farms tested in our study may have benefitted from beginning vaccination regimens in gilts several weeks before the initiating dose in their current protocols, or vaccinating at the multiplier site, in order to maximize the vaccine response to all antigens contained in the multi-valent vaccines. Replacement gilts play a significant role in affecting the overall health of the breeding herd and serological monitoring utilizing both NP ELISA and HI assays to evaluate IAV antibody status can help reduce the propagation of new and endemic IAV in the breeding herd.

### **Conflicts of Interest**

The authors declare that there are no conflicts of interest.

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**Authors' Contributions**

JSC processed the samples, performed the serological testing, and wrote the manuscript. ALV helped select viruses for testing and wrote the manuscript. JQZ wrote the manuscript. PCG conceived and acquired funding for the project, contacted and recruited producers, and wrote the manuscript. All authors have read and approved the final manuscript.

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Table 1: Experimental design and serum sample collection schedule.

Population location	Group	Mean #. of samples per farm <sup>a*</sup>	Total # of samples	Gilt age or category	Time between vaccine & collection	Time frame <sup>b†</sup>
GDU <sup>c‡</sup> /Isolation/Acclimation	Pre-Vac	42 (40-50)	504	20-28 weeks	N/A	Oct 2015 - Jan 2016
GDU/Isolation/Gestation	Post-Vac	30 (20-44)	325	28-32 weeks	2-4 wks post vac	Dec 2015 - Jul 2016
Breeding Herd	Post-Int 1	23 (14-35)	251	Parity 1	4-6 wks post vac	Mar 2016 - Dec 2016
Breeding Herd	Post-Int 2	20 (7-37)	213	Parity 2	4-6 wks post vac	Sep 2016 - Mar 2017

\*: Mean number and range of serum samples collected from gilts per farm.

†: Time range when sample collection occurred.

‡: Gilt developer unit.

Table 2: Farm demographic information organized into Gilt Source, Sow Farm, and Vaccination demographics

Region	N	Sow Farm State	Gilt Source Demographics				Sow Farm Demographics			Vaccination Demographics					
			State	Multiplication	IAV status		Population - sows	Population - gilts	Age*	Isolation protocol	Vaccine antigen	Vaccine Type	Age at Entry to Breeding herd†	# of Doses	Time of vaccination‡
1	40	PA	ND	External	Offsite	Negative	1550	650	24	None	Farm-specific	Whole virus	24-26	2	0 & +2 weeks§
	50	NC-1	VA	External	Offsite	Negative	3696	814	28	None	FluSure XP®	Whole virus	32	2	-6 & -4 weeks
	41	NC-2	MS	External	Offsite	Positive	3085	872	25	None	FluSure XP®	Whole virus	30-32	2	-5 & 0 weeks
2	43	IA	ND	External	Offsite	Negative	2800	375	21	8-10 weeks	Farm-specific	Subunit	30	2	-6 & -4 weeks
	40	IN	ND	External	Offsite	Negative	1600	300	24	4 weeks	FluSure XP®	Whole virus	28	2	-4 & -1 weeks
	40	IL	IL	Internal	Onsite	Positive	5800	800	20	8 weeks	FluSure XP®	Whole virus	30	2	-7 & 0 weeks
3	40	OK	OK	Internal	Offsite	Positive	63000	3150	20	N/A	N/A	N/A	N/A	N/A	N/A
	50	TX	TX	Internal	Onsite	Positive	1984	1280	28	None	MaxiVac Excell™	Whole virus	30-32	2	-4 & 0 weeks
	40	MO	IL	External	Offsite	Positive	2360	185	23	8 weeks	FluSure XP®	Whole virus	30-32	2	-7 & -3 weeks
4	40	KS	KS	Internal	Offsite	Positive	1250	145	24	None	Farm-specific	Subunit	33	2	-9 & -6 weeks
	40	NE	NE	Internal	Offsite	Positive	5600	300	22	None	Farm-specific	Subunit	26	1	-4 weeks
	40	ND	ND	Internal	Onsite	Negative	4000	400	26	N/A	N/A	N/A	26	N/A	N/A

\*: Age in weeks at sample collection

†: Age at herd entry in weeks.

‡: Timing of vaccination relative to entry into the main breeding herd (0 weeks).

§: Vaccine administered post-entry into the herd.

Table 3: Influenza A Viruses used for Representative Antigens, Homologous Vaccine Antigens, and Putative Vaccine Antigens for HI assay testing

	Subtype	Reference	Year	Virus	Cluster/Clade	GenBank No.
Representative antigens	H1N1	H1-γ	2007	A/Swine/Ohio/511445/2007	Gamma	EU604689
	H1N2	H1-δ1b	2013	A/Swine/South Dakota/A01349341/2013	Delta-1b	KC844209.1
	H1N1	H1-δ2	2005	A/Swine/Illinois/00685/2005	Delta-2	CY081899.1
	H3N2	H3 Red	2011	A/Swine/New York/A01104005/2011	Cluster IVA	JN940422.1
	H3N2	H3-Green	2014	A/Swine/Iowa/A01480656/2014	Cluster IVA	KJ635928.1
	H3N2	H3 hu-like	2014	A/Swine/Missouri/A01410819/2014	2010 human-like	KJ941380.1
FluSure XP Vaccine Antigens	H1N1	H1-γ	2008*	FluSure XP 012	Gamma	N/A
	H1N2	H1-δ1	2008*	FluSure XP 726	Delta-1	N/A
	H1N1	H1-δ2	2008*	FluSure XP 031	Delta-2	N/A
	H3N2	H3-IV	2011*	FluSure XP 069	Cluster IV	N/A
MaxiVac <b>putative</b> vaccine antigens	H1N1	H1-β	2008	A/Swine/Kentucky/02086/2008	Beta	N/A
	H1N1	H1-γ	2008	A/Swine/Ohio/02026/2008	Gamma	N/A
	H1N2	H1-δ2	2010	A/Swine/Iowa/02955/2010	Delta-2	N/A
	H3N2	H3-IV	2009	A/Swine/Minnesota/02782/2009	Cluster IV	N/A
IA farm <b>putative</b> vaccine antigens	H1N2	H1-δ1	2013	A/swine/Illinois/A01291356/2013	Delta-1	AGX00886.1
	H1N1	H1-γ	2014	A/swine/Iowa/A01410104/2014	Gamma	KJ437539.1
	H3N2	H3 hu-like	2015	A/swine/Iowa/A02076444/2015	Human like	KR812416.1
KS farm <b>putative</b> vaccine antigens	H1N1	H1-γ	2011	A/swine/Indiana/41733/2011	Gamma	N/A
	H1N2	H1-δ2	2014	A/swine/Missouri/A01411322/2014	Delta-2	KJ437545.1
	H3N2	H3-IV	2009	A/swine/Minnesota/02782/2009	Cluster IV	N/A
	H1N1	H1-pdm09	2009	A/California/04/2009	Pandemic 2009	N/A
PA farm vaccine antigens	H1N1	H1-γ	2013	A/swine/Ohio/47242/2013	Gamma	N/A
	H1N1	H1-δ1	2012	A/swine/Indiana/2012	Delta-1	N/A
	H1N2	H1-δ2	2015	A/swine/Pennsylvania/16577/2015	Delta-2	N/A
	H3N2	H3-IV	2015	A/swine/19299/2015	Cluster IV	N/A
	H3N2	H3-IVB	2015	A/swine/Michigan/18353/2015	Cluster IVB	N/A
NE farm <b>putative</b> vaccine antigens	H1N1	H1-δ2	2014	A/swine/North Carolina/A01477778/2014	Delta-2	KP186042.1
	H1N1	H1-pdm09	2014	A/swine/Kansas/A01410327/2014	Pandemic 2009	KJ605091.1
IL farm vaccine antigens	H1N1	H1-pdm09	2016	A/swine/Illinois/A01729364/2016	Pandemic 2009	KU598287.1
	H1N2	H1-δ1	2015	A/swine/Illinois/A01797385/2015	Delta-1	KU301001.1
	H3N2	H3 hu-like	2015	A/swine/Missouri/61110/2015	Human like	N/A

\*: Dates are based on when Zoetis distributed the antigens to the ISU VDL for use in diagnostic testing. According the ISU VDL, all antigens are currently used in the vaccine product.

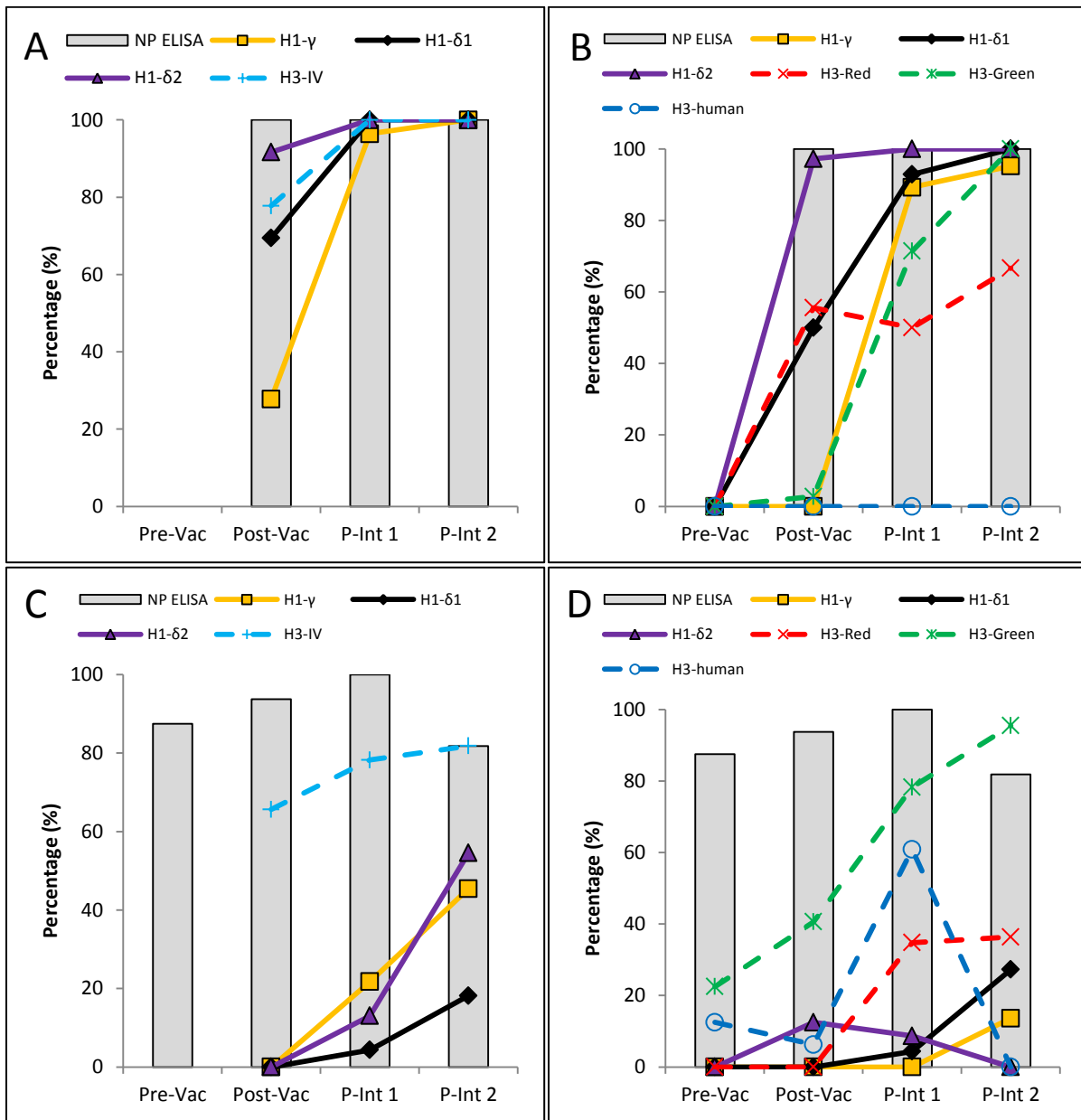


Figure 1: Graphs of the Percent NP ELISA Antibody Positive and Percent HI Antibody Positive Replacement Gilts at Pre-Vac, Post-Vac, P-Int 1, and P-Int 2 to Homologous Vaccine Virus Antigens (A, C) and Six Representative Virus Antigens (B, D) for the NC-2 (A, B) and MO (C, D) farms. Note both farms used the FluSure vaccine.

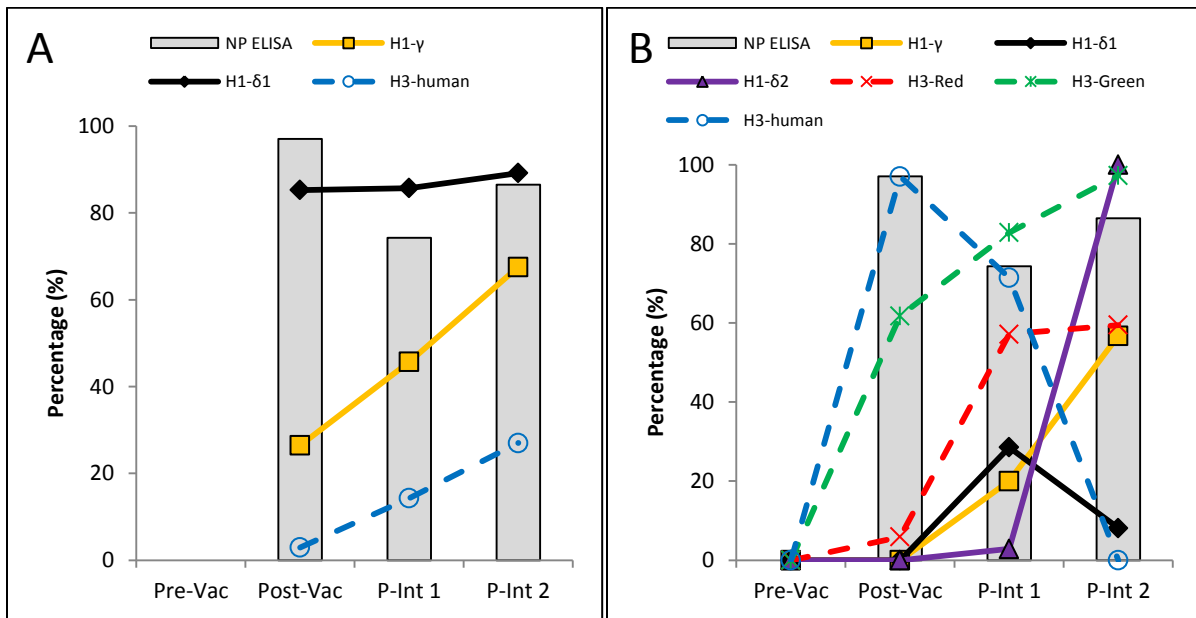


Figure 2: Graphs of the Percent NP ELISA Antibody Positive and Percent HI Antibody Positive Replacement Gilts at Pre-Vac, Post-Vac, P-Int 1, and P-Int 2 to Homologous Vaccine Virus Antigens (A) and Six Representative Virus Antigens (B) for the IA (A, B) farm. Note the IA farm used a subunit HA replicon particle vaccine.

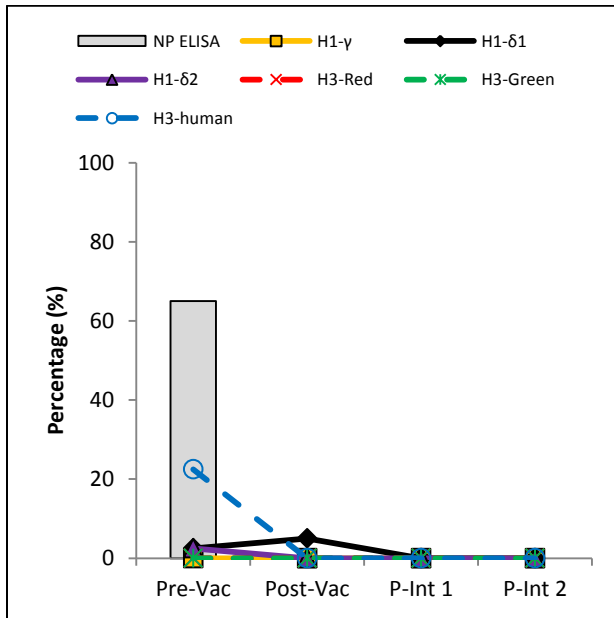


Figure 3: Graphs of the Percent NP ELISA Antibody Positive and Percent HI Antibody Positive Replacement Gilts at Pre-Vac, Post-Vac, P-Int 1, and P-Int 2 to Six Representative Virus Antigens for the ND farm. Note the ND farm did not vaccinate.

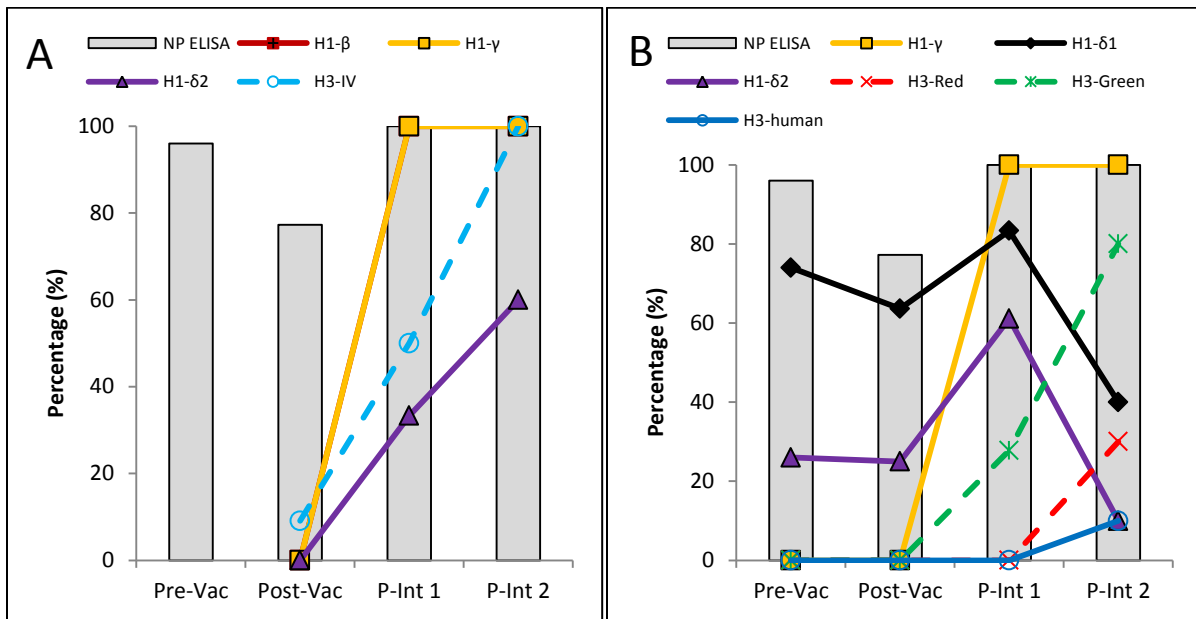


Figure S1: Graphs of the Percent NP ELISA Antibody Positive and Percent HI Antibody Positive Replacement Gilts at Pre-Vac, Post-Vac, P-Int 1, and P-Int 2 to Homologous Vaccine Virus Antigens (A) and Six Representative Virus Antigens (B) for the TX (A, B) farm. Note the TX farm used the MaxiVac Excel vaccine.

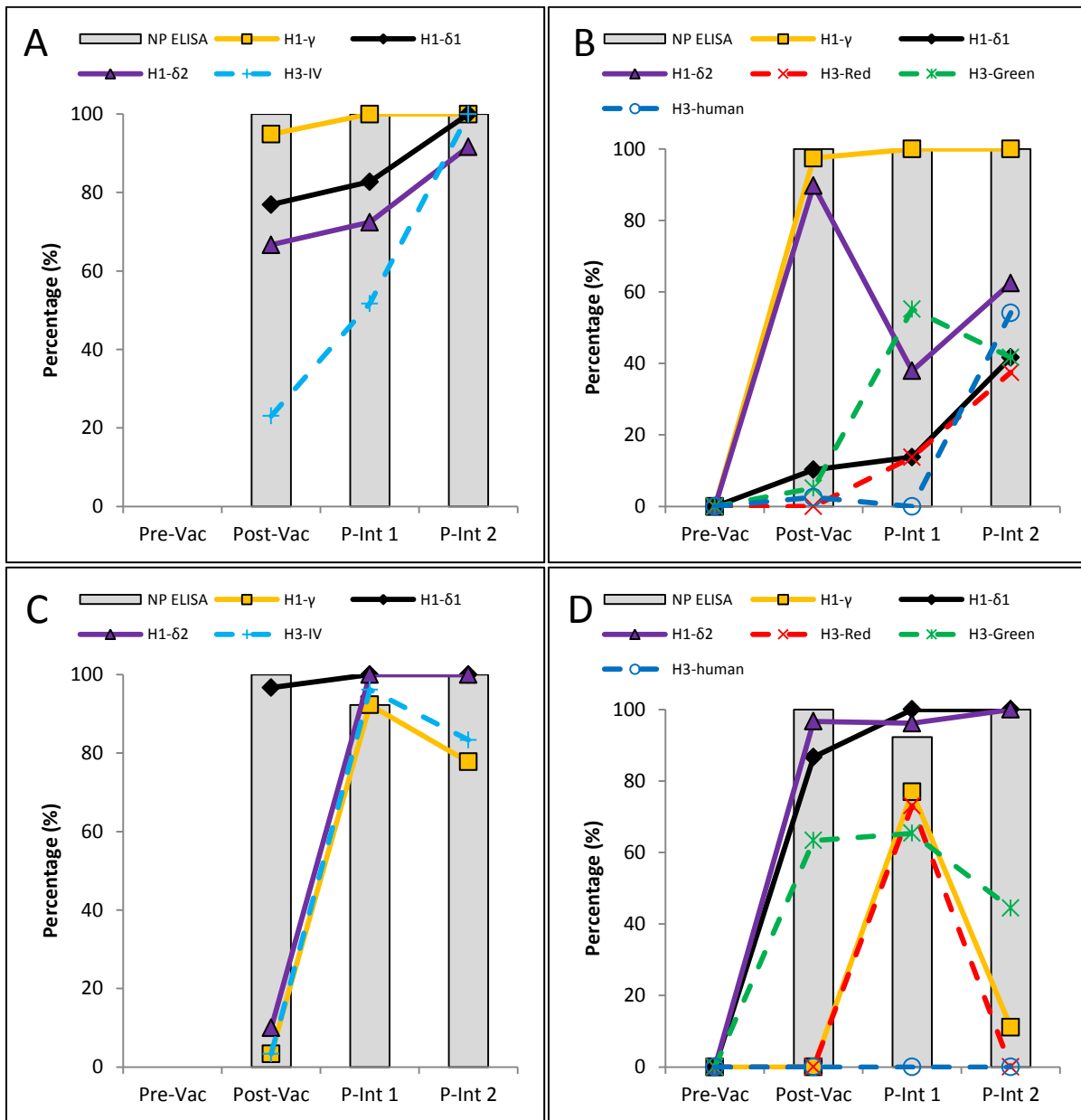


Figure S2: Graphs of the Percent NP ELISA Antibody Positive and Percent HI Antibody Positive Replacement Gilts at Pre-Vac, Post-Vac, P-Int 1, and P-Int 2 to Homologous Vaccine Virus Antigens (A, C) and Six Representative Virus Antigens (B, D) for the IN (A, B) and NC-1 (C, D) farms. Note both farms used the FluSure vaccine.



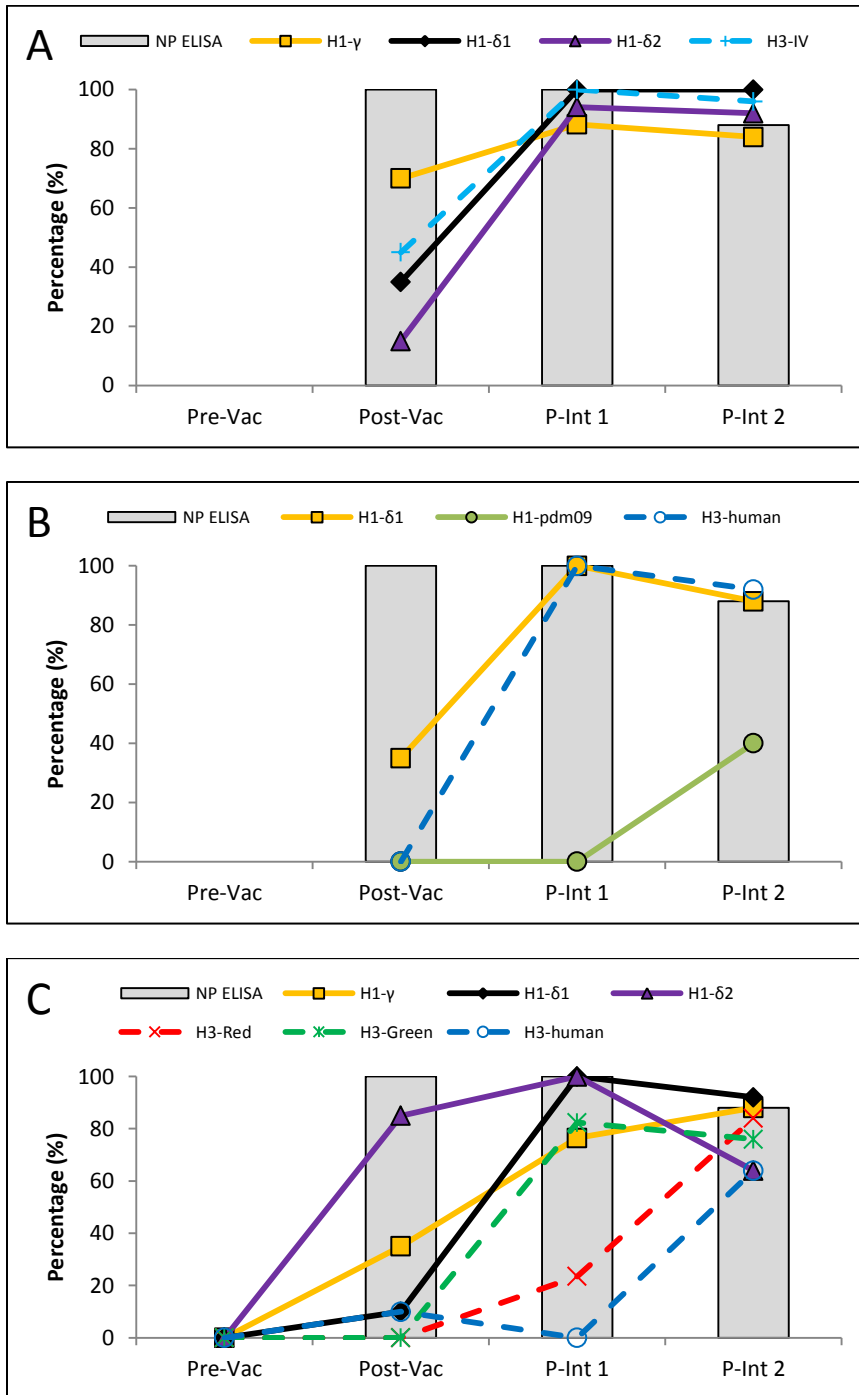


Figure S3: Graphs of the Percent NP ELISA Antibody Positive and Percent HI Antibody Positive Replacement Gilts at Pre-Vac, Post-Vac, P-Int 1, and P-Int 2 to Homologous Vaccine Virus Antigens for FluSure (A) and Farm Specific (B) plus Six Representative Virus Antigens (C) for the IL (A, B, C) farms. Note the IL farm switched from FluSure vaccine to Farm Specific vaccine between P-Int 1 and P-Int 2.

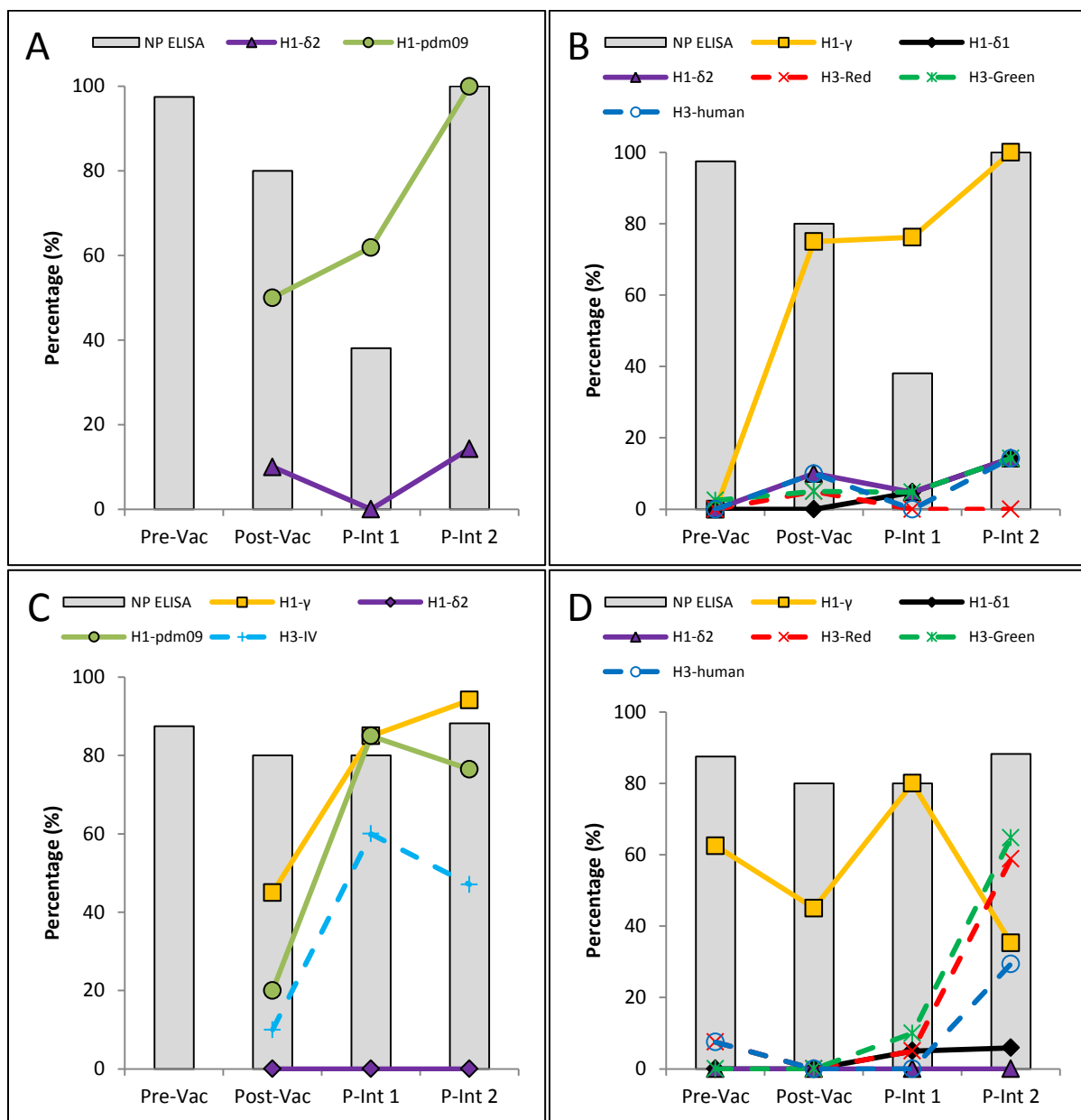


Figure S4: Graphs of the Percent NP ELISA Antibody Positive and Percent HI Antibody Positive Replacement Gilts at Pre-Vac, Post-Vac, P-Int 1, and P-Int 2 to Homologous Vaccine Virus Antigens (A, C) and Six Representative Virus Antigens (B, D) for the NE (A, B) and KS (C, D) farms. Note both farms used a subunit HA replicon particle vaccine.

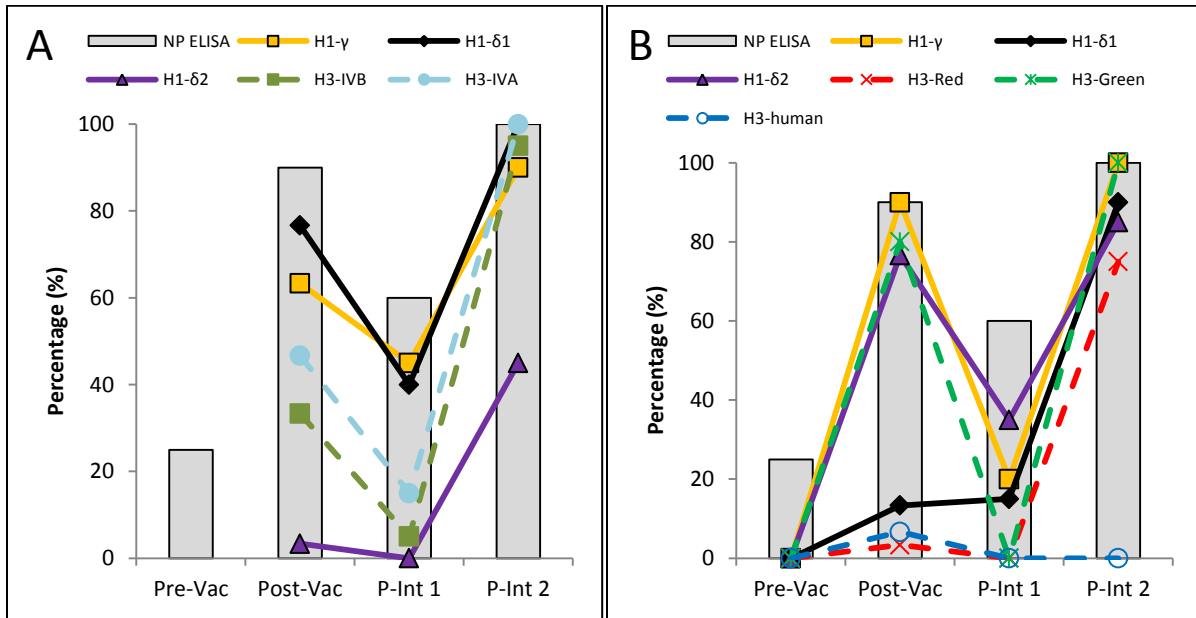


Figure S5: Graphs of the Percent NP ELISA Antibody Positive and Percent HI Antibody Positive Replacement Gilts at Pre-Vac, Post-Vac, P-Int 1, and P-Int 2 to Homologous Vaccine Virus Antigens (A) and Six Representative Virus Antigens (B) for the PA (A, B) farm. Note PA farm used a farm specific whole inactivated virus vaccine.

## CHAPTER 4. SUMMARY AND CONCLUSIONS

The objective of this project was to determine the serological status of new replacement gilts pre- and post- vaccination. This goal was accomplished by enrolling twelve farms from across the United States into the study. Serum was collected at four time points from the same subpopulation of replacement gilts on each farm. Antibody response was evaluated using the Nucleoprotein Enzyme-linked Immunosorbent Assay (NP ELISA) and a Hemagglutination Inhibition (HI) Assay using homologous vaccine antigens and six H1 and H3 antigens representing the predominant phylogenetic clusters and antigenic clades circulating in US swine.

The literature review chapter for this thesis revealed two important gaps in our knowledge of IAV in swine. First, there was a lack of recent IAV serological data on swine in the US. Serological studies have been conducted previously, but are considered historic and may lack data relevant to contemporary IAV in swine. Current epidemiological evaluations of IAV in US swine have focused on virus isolation and characterization of the genetic and antigenic diversity. Second, there is a lack of IAV information regarding new replacement gilts within US production systems. The majority of scientific studies focus on mature sows, nursing piglets, or finisher swine when evaluating disease dynamics. New replacement gilts appear to be an understudied group. This thesis sought to address both knowledge gaps by targeting new replacement gilts to evaluate their IAV antibody status during a critical time in their development and preparation to enter the breeding farm. Chapter 3 and 4 detail the results from the serological evaluation of new replacement gilts divided into pre- and post-vaccination data, respectively.

Chapter 3 pre-vaccination data suggested the IAV dynamics of the multiplication or source herd may determine the IAV antibody status of new replacement gilts. Additionally, the data demonstrated a number of new replacement gilts are entering the farm IAV antibody naïve, which could leave them susceptible to resident endemic viruses and contribute to maintenance of endemic infections. Increased IAV antibody surveillance of replacement gilts could improve the ability of breeding farms to adequately prepare gilts to enter the breeding herd. Care should be taken when choosing a serology test and interpretation of the data due to the increased genetic diversity of IAV-S in US swine and how that can affect test outcomes. The NP ELISA is the preferred diagnostic assay for evaluating potential IAV exposure.

The post-vaccination data demonstrated the number of IAV antibody positive gilts is variable across farms and over time regardless of using commercial or farm specific vaccines. Several farms had gilts without detectable IAV antibody responses or low numbers of gilts with homologous IAV positive HI antibody titers even after multiple doses of vaccine. This lack of antibody response suggests a gap in protection based on the assays performed. The data also revealed the potential limitations of the antibody assays. The HI assay is a powerful tool for evaluating homologous vaccine responses and correlates with protection, and works well to evaluate cross-reactive heterologous antibody responses if the antisera and antigen have been characterized. The NP ELISA can be an excellent tool for evaluating potential IAV exposure but is unable to detect certain types of vaccine platforms (for example, replicon particle vaccines) and does not correlate with protection. Vaccination of breeding age gilts is important for inducing maternal antibodies to protect suckling piglets.

Increased antibody surveillance is recommended to help monitor IAV vaccine immune responses as well as potential cross-reactive antibody that may be present in breeding gilts.

Collectively, it can be concluded from this thesis that IAV vaccine antibody responses in the field are highly variable, unpredictable with diagnostic assays, and require increased surveillance to ensure protective levels are maintained. If the results of this thesis are reflective of the swine industry as a whole, then changes in vaccination protocols could be considered. The update of current vaccines with contemporary viruses or the adoption of novel vaccine platforms may help increase protection in US swineherds. Regardless of any changes made to vaccination protocols, increased serological surveillance would help the industry. Increased surveillance would help identify gaps in protection and allow for the evaluation of any changes to vaccination protocols.

There are a number of possible future projects based on the results of this thesis. One line of future work could be going back and reevaluating some of the serum from some of the more interesting farms. The North Dakota herd was located in a region with low-density of swine and did not vaccinate their swine, yet there were a large number of NP ELISA positive animals and several samples tested HI antibody positive for the H3hu-like representative antigen. It would be interesting to go back and retest the serum with additional representative viruses such as more swine H3 hu-like viruses and some human seasonal H3 IAV. This additional testing could help investigate if a human virus infected swine. A second avenue of future research could be performing a second longitudinal study and combine virological and serological methods. Another set of farms could be sampled over a year and oral fluids or nasal swabs could be collected in addition to serum samples. Utilizing virology tests, such as virus isolation and sequencing could be performed in addition to serological testing. This

would allow for the correlation of viral infection and antibody profile and we could examine how effective vaccination is at preventing endemic viral circulation.